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CCXIV. THE LARGE SCALE PREPARATION OF ASCORBIC ACID FROM HUNGARIAN PEPPER (*CAPSICUM ANNUUM*)[1].

By I. BANGA and ALBERT SZENT-GYÖRGYI.

From the Institute of Medical Chemistry, University of Szeged, Hungary.

(*Received July 14th, 1934.*)

Two main varieties of *Capsicum* are cultivated on the Hungarian plains: the smaller and drier "noble" type, used in dry powdered condition for spicing, and the fleshier type, which is eaten fresh. Both types have many sub-varieties. All the varieties tested seem to be equally suitable for the preparation of ascorbic acid. The juice of all varieties, titrated with acid iodine, uses 2–3 ml. 0·01 *N* iodine per ml., which reduction is mainly due to ascorbic acid (2–2·5 mg./ml.). On the whole "noble" pepper titrates somewhat higher than the fleshy varieties but gives less juice.

For the production of ascorbic acid only the fruit is used. The stem, core and seeds—forming about one-third of the total weight—are rejected, and the flesh is minced. Unripe, green pepper contains little ascorbic acid. The titre rises rapidly with ripening, when the green fruit turns first brown and then red. In the brown fruit the titre has almost reached maximum values. When the ripe, red fruits begin to shrink, dry or liquefy the titre falls again. Some varieties do not turn red, but become yellow on ripening. Their ascorbic acid content is equally high.

To every 450 litres of the pulp 22 kg. acid lead acetate are added in solution. This solution is prepared by dissolving 22 kg. plumbum aceticum in 9 litres water and adding 170 ml. 85 % formic acid. The pulp is thoroughly minced and pressed out on a fruit press. 450 litres of minced noble paprika leave about 100 kg. residue, 450 litres fleshy pepper leave 70 kg. residue. The juice, which leaves the press as a limpid fluid, can be stored for several days without loss in filled, stoppered bottles.

To every 10 litres of the juice are added 500 ml. of a lead acetate solution prepared by dissolving with the aid of heat 1 kg. plumbum aceticum in 1 litre of water. Ammonia is then added in a thin stream and stirred in till phenolphthalein, dropped on the surface, gives a faint colour, or bromothymol blue indicates a slightly alkaline reaction. The precipitate is separated quickly and sharply on suitable centrifuge. A Sharples supercentrifuge was used in our laboratory (big industrial type) with much success. 40 litres of juice yield on an average 5 kg. wet lead precipitate. This precipitate contains about half of the ascorbic acid originally present in the fruit. It can be stored for several days without loss of vitamin if air is excluded. This precipitate is well suited for the transport of the crude substance. The lead precipitate is then suspended in the smallest possible volume of water, strong hydrochloric acid is added to the well stirred mass till the fluid just begins to redden thymol blue. The $PbCl_2$ is separated on a centrifuge and washed with a little water. The aqueous

[1] This Research was sponsored by the Josiah Macy Jr. Foundation, New York.

fluids are united and concentrated under reduced pressure to a syrupy fluid, containing not more than 15–20 % water. It is very essential that this concentration should take place at a low temperature and in a short time. On heating the juice turns brown, and resinous matter appears which makes further work very difficult. We applied with much success a special vacuum-distilling apparatus, in which heating and distillation took place in a spiral tube. The time of heating of every part of the fluid was limited to the time necessary for the fluid to run through this spiral (1–2 minutes). Concentration was effected in two steps. Contact with copper should be avoided throughout the whole preparation.

The resulting light brown syrupy fluid contains 10 % ascorbic acid, calculated on the dry weight of the residue. In this form the vitamin is stable and can be stored in a cool place for weeks without loss of activity.

To this syrupy fluid an equal volume of anhydrous acetone is added and the mixture is strongly shaken mechanically for ten minutes. It is essential that the shaking should be very energetic and should lead to a good dispersion of the phases. The mixture is then allowed to settle for 15 minutes and the acetone phase poured off. The remaining oil is again shaken with an equal volume of acetone. After this second shaking the suspension usually does not separate, and it is necessary to effect a separation on a centrifuge. The acetone is again poured off and the residue treated twice more in the same manner. At the 3rd and 4th shakings the phases separate spontaneously. The 3rd and 4th acetone extracts may be used to extract the next batch of fluid.

The united acetone fluids contain the greater part of the vitamin, about one-fifth of the total vitamin remaining in the acetone-extracted residue. Part of this can be extracted by shaking the oil with 96 % ethyl alcohol. The extract is freed from alcohol by distillation, and the residue is then extracted with acetone or added to the next batch of syrup.

The acetone fluid contains 25 % vitamin calculated for dry weight. For its further treatment three different methods were applied with equal success. The yield in all the three is about equal, a quarter of the vitamin originally present in the plant being obtained in crystalline condition. The simplest method, given in the first place, yields good results if the extract to be treated has a relatively high vitamin content, almost 25 %. With weaker extracts the alkali method gives better results. The lead method involves the most labour.

Acetone method. The acetone extract is concentrated under reduced pressure and low temperature to a sticky syrup, which is treated on the shaking machine with dry acetone as described above. The greater part of the vitamin passes into the acetone phase, which contains 30–40 % vitamin calculated for dry weight.

The oily residue is dissolved in a little ethyl alcohol and again precipitated with excess of acetone, use being made of the shaking machine. This secondary acetone extract is concentrated and the resulting oil treated with pure acetone.

The acetone extracts are united and concentrated *in vacuo* to a smaller volume. To this concentrated acetone extract 1 ml. of *n*-butyl alcohol is added for every g. of vitamin present. The rest of the acetone is then distilled off under reduced pressure. The vitamin is thus left dissolved in butyl alcohol as a sticky syrup, which on cooling soon begins to crystallise. Crystallisation is allowed to proceed for 2–3 days at 0°.

Alkali method. To the acetone extract cold saturated aqueous NaOH solution is added in small portions with very energetic mechanical shaking. The Na salt of the vitamin separates in the form of a dark brown liquid phase. The addition of alkali is continued till 1 ml. of the supernatant fluid does not require more than

0·6 ml. of 0·01 *N* iodine in acid solution, starch being used as indicator. Excess of alkali is to be avoided.

The acetone is syphoned off and the phase containing the vitamin is tested with phenolphthalein. If it does not colour the indicator, NaOH is added till the reaction is alkaline. Then an equal volume of 96 % ethyl alcohol is added to the fluid, and the mixture is shaken up and allowed to separate. The alcohol is poured off and the residue shaken out once more with the same solvent. The alcohol takes out only relatively little vitamin, using up no more than 1 ml. iodine per ml. on titration.

A further quantity of alcohol is added and then strong hydrochloric acid in small portions till the fluid just begins to change the colour of thymol blue; thus the total Na is neutralised. More alcohol is then added until five parts of alcohol are present for every part of HCl used. The fluid is set aside for the night at $-20°$, the NaCl being allowed to crystallise. Next day the NaCl crystals are separated on a Büchner funnel and washed with alcohol.

The alcoholic filtrate is concentrated under reduced pressure to a syrup, and to this acetone is added in small portions. The first quantities of acetone mix with the fluid, but the excess produces a bulky oily precipitate, which is repeatedly shaken out with acetone. The acetone extracts are united, the acetone partly distilled off under reduced pressure, *n*-butyl alcohol added as above, distillation continued and crystallisation effected.

Acid lead method. Lead acetate is melted and heated to boiling. Then to every kg. of lead acetate 1 litre of 96 % ethyl alcohol is added. This hot solution is added in a thin stream to the above acetone extract with vigorous stirring. The vitamin separates in form of a resinous mass. Lead acetate is added, till the titre of the fluid does not fall on further addition of the reagent. The final titre of the fluid is about 0·6 ml. 0·01 *N* iodine per ml. For every litre of acetone extract 300–400 ml. of lead acetate solution are used.

The resinous precipitate is separated and decomposed in a mortar by strong HCl, the acid being added in small portions, as fast as it is used up, till the fluid just begins to redden thymol blue faintly but permanently. The $PbCl_2$ is separated, the precipitate washed with a little water on the centrifuge, and the watery extracts are concentrated *in vacuo* to an oil, which is extracted with acetone as above and crystallised from *n*-butyl alcohol.

Crystallisation, etc. The crystals of ascorbic acid can be separated on a Büchner funnel, but this process is usually very tedious owing to the viscosity of the mother-liquor. It is preferable to separate the crystals on the centrifuge, pour off the mother-liquor and then suspend the crystals in a small volume of acetone, to which enough methyl alcohol is added to prevent the formation of a precipitate (mostly 8 : 2). This suspension is filtered on a Büchner funnel and washed by the same alcohol-acetone mixture. The crystals are again suspended and washed with acetone, to which the necessary quantity of methyl alcohol is added. In the later washings less methyl alcohol is needed.

It has been found that the mother-liquor of any crystallisation always contains 20 % of vitamin, calculated for dry weight. If the crystallisation is induced in a fluid containing 30 % vitamin (for dry weight), only one-third of the vitamin comes out in crystals. From a fluid with 40 % vitamin, one-half of the ascorbic acid crystallises. It is very advisable to control the vitamin content and relative purity of the fluids at every step of the preparation.

The mother-liquor from the crystallisation can be fractionated once more by concentrating it to a sticky oil and extracting this with acetone on the shaking machine. Still better results are obtained by treating the mother-liquor with

NaOH and repeating the whole procedure described as the alkali method. This second fractionation yields about half as much crystalline vitamin as the first crystallisation. Further attempts to recover more of the vitamin after the second crystallisation were unsuccessful. The loss of vitamin in the mother-liquors is quite substantial and it is felt that improvements on this point are necessary and possible.

In dry recrystallised form the vitamin is stable even at room temperature.

Recrystallisation was effected in the following way. Methanol and dioxan were mixed in the proportion of 4 : 1 and in this mixture a saturated solution of vitamin was prepared by boiling. For every kg. of the vitamin about 6 litres of fluid are needed. The liquid was then concentrated under reduced pressure till strong crystallisation and bumping made further concentration difficult. The vitamin, originally yellow, now separated in the form of colourless crystals with the correct M.P. The suspension was set aside for the night in the ice-chest and filtered on a Büchner funnel, the mother-liquor being concentrated further and again allowed to crystallise.

3 kg. of pure ascorbic acid were prepared in our laboratory by the above methods.

No patents were taken out for the process.

CCXV. A NOTE ON THE USE OF TARTRAZINE IN THE DETERMINATION OF CHLORIDES IN BIOLOGICAL MATERIAL.

By WILLIAM ROBERT FEARON and WILLIAM ALEXANDER GILLESPIE.

From the Department of Physiology, Trinity College, Dublin.

(*Received July 16th, 1934.*)

The list of adsorption indicators available for determining the end-point in the titration of halides by silver nitrate has been extended recently by Berry and Durrant [1930; Berry, 1932], who have introduced tartrazine (tartar yellow or tartrazole yellow) and phenosafranine for titrations carried out in acid solutions.

We have found the first of these particularly useful for the estimation of chloride in biological secretions and extracts. It is a convenient indicator for general class work and also provides a simple demonstration of the phenomenon of adsorption.

Direct method.

This is suitable for the estimation of chloride in normal urine and similar protein-free solutions, including sea water.

Method. Ten ml. of 0·1 *N* $AgNO_3$ in a large test-tube are acidified with 5 drops of pure HNO_3, and 2–5 drops of 0·5 % aqueous tartrazine solution are added.

The urine is run in from a burette in quantities of 0·1–0·5 ml. at a time, the mixture being well shaken after each addition. The precipitate of silver chloride which first forms is coloured buff by the adsorbed indicator, and separates out from an almost colourless solution. The end-point is shown by the sudden release of the adsorbed indicator, which passes from precipitate to solution, leaving the precipitate pure white and colouring the solution greenish yellow. This change is very sharp, and may be evoked by a single drop of urine or of a 0·1 % solution of NaCl.

Indirect method.

This is suitable where only small quantities of the chloride solution are available, as in the titration of cerebrospinal fluid, or where the solution contains proteins or other colloids that interfere with the aggregation of the silver chloride precipitate.

Method. One ml. of the chloride solution is added to the mixture of 10 ml. 0·1 *N* $AgNO_3$, acid and indicator, prepared as already described. After shaking well, the mixture is titrated with previously standardised 0·1 *N* HCl.

Notes on the method.

Where the chloride concentration is less than 0·1 % NaCl it is advisable to use not more than two drops of indicator, as the precipitated silver chloride may be insufficient in amount to adsorb the excess of tartrazine. Working with minimum quantities of indicator it is possible to evoke the end-point by addition

of as little as 0·05 mg. NaCl to the titration mixture. This method may also be adopted for determining the chloride contamination of the HNO_3 used. The tartrazine is liable to be contaminated by chloride, but the quantity present is usually insignificant. An important advantage of the method lies in the double colour change occurring at the end-point, namely (1) the change in the colour of the precipitate from buff to white, and (2) the change in the supernatant liquid from colourless to greenish yellow. By using the optimum of indicator both these changes are observed simultaneously, and the end-point is sharp and very sensitive. When proteins or other protective colloids are present the aggregation of the silver chloride may be prevented, and it becomes necessary to rely entirely on the colour change shown by the precipitate. Thus, in titrating by the direct method such liquids as albuminous urine, saliva, gastric juice, or even milk, moderately satisfactory results may be obtained by using an excess (8–10 drops) of indicator and matching the colour-change in the mixture with a control that has exceeded the end-point. The indirect method, however, is much more satisfactory for such liquids.

Proteins also interfere with the accuracy of the result by combining to a certain extent with the silver reagent and lowering its chloride value. This objection applies equally to the other argentimetric methods used for the titration of chlorides in biological material, and when possible the proteins should be removed by heat-coagulation or precipitation before the estimation. The commoner solutes in pathological urine, such as acetone, acetoacetic acid and glucose, do not interfere with the method. Bile pigment obscures the end-point but may be removed by bleaching the urine with a little persulphate or nitrite, after addition of a drop of nitric acid. Proteins cause difficulty. Sometimes the urine may be deproteinised satisfactorily by heat-coagulation after addition of sodium acetate and acetic acid, the coagulum being washed subsequently with hot water to remove chlorides, and the filtrates combined and made up to known volume. It is frequently found, however, that when the urine has become infected the protein undergoes partial bacterial decomposition and is no longer completely removed by heat-coagulation.

The standardisation of the silver nitrate and other reagents can be accomplished most readily by using the standard sea water of known chlorine content (usually 19·386 ‰) issued by the International Committee for Exploration of the Sea, Laboratoire Hydrographique, Polyteknisk Laereanstalt, Copenhagen.

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CCXVI. STUDIES IN THE CHOLESTEROL CONTENT OF HUMAN PLASMA.

IV. THE NON-STEROL UNSAPONIFIABLE MATTER OF HUMAN PLASMA.

By JOHN ADDYMAN GARDNER and HUGH GAINSBOROUGH.

From the Biochemical Laboratory, St George's Hospital, London.

(*Received July 18th, 1934.*)

Some years ago Wacker [1912] noted the presence of unsaponifiable matter other than sterols in tissue extracts. He called this material "Begleitsubstanz des Cholesterins" and stated that it gave sterol colour reactions in modified manner. In our analyses of plasma-cholesterol we have always recorded the amount of this non-sterol unsaponifiable matter which we refer to as N.S. Its amount, reckoned as if present in the plasma, varies between 0·15 and 1·2 %. This amount is quite unrelated to the sterol content whether free, combined as ester or total. In the course of these analyses we accumulated about 10 g. of this material and it seemed worth while to submit it to closer investigation. We found that it was easily separable by means of light petroleum into an insoluble portion A and a readily soluble portion B. In individual analyses these portions A and B bore no definite relation to each other.

The insoluble portion A was soluble in alcohol, ether or chloroform and on evaporation it dried to a resin or brittle scale. In chloroform solution with acetic anhydride and sulphuric acid it gave a brownish coloration which sometimes attained a dirty green tinge. It was not volatile in superheated steam nor could it be distilled in a high vacuum. It contained traces of nitrogen and sulphur but no phosphorus. We found that by heating sodium ethoxide in alcoholic solution with ether under a reflux, in similar manner to our usual saponification procedure, the N.S. obtained consisted of material resembling the A portion. This and other experiments suggested that this portion was produced by condensation of the impurities in our reagents with one another and possibly with constituents of plasma. We then made a series of plasma analyses using carefully purified alcohol and ether, when this constituent of the N.S. practically vanished. We therefore did not consider that it merited further investigation.

The portion B soluble in light petroleum was entirely volatile in superheated steam; it could be distilled in high vacuum without decomposition at a temperature of 150 to 220°/1 to 2 mm. After evaporation from light petroleum it was left as a vaseline-like substance and on fractional crystallisation from alcohol, methyl alcohol, ethyl acetate or acetone it yielded crystalline paraffins and also hydrocarbons of liquid consistency. Three different crystalline specimens had melting points 56–57°, 62–63°, and 63–64°. Analysis of representative specimens yielded the following results.

1	H 14·40 %	C 85·35 %	Mol. wt. (Rast)	496·5
2	H 14·66 %	C 85·37 %	,,	438
3	H 14·64 %	C 85·21 %	,,	340·5

These were obviously hydrocarbons. Some specimens however seemed to contain traces of oxygen suggesting the presence of some fatty acid or higher alcohol *etc.*

It seemed to be highly unlikely, in view of the generally accepted knowledge of the composition of blood-plasma, that paraffins in such quantities as we found could be real constituents of the plasma, and therefore it seemed probable that they were artefacts. We thought that the vaseline used to grease the stopcocks of the separating funnels might have been extracted in our analyses in sufficient quantity to yield these results. Though unlikely we also considered whether liquid paraffin [Channon and Collinson, 1929] frequently administered to hospital patients, could be a source of the N.S. in the blood. We therefore took the hospital liquid paraffin and the laboratory vaseline and redistilled them fractionally in high vacuum as we did the plasma N.S. The paraffinum liquidum yielded only liquid fractions which would not solidify even on freezing, and which appeared to consist of hydrocarbons of the naphthene series. They were quite unlike the material obtained from the plasma N.S. and hence could be eliminated. The vaseline however readily separated into a series of fractions which solidified on cooling and when crystallised from alcohol or acetone yielded hydrocarbons physically precisely similar and of similar composition to those described above. It therefore seemed likely that the tap vaseline was a source of the hydrocarbons in our plasma N.S. It was found that we could get such grease into solution in quantities comparable with those obtained in our blood analyses by extracting dilute caustic soda with ether in separating funnels, using quantities such as would correspond with a definite number of our usual analyses. In order to test whether the whole of the N.S. soluble in light petroleum was of this origin we made a number of plasma analyses with carefully purified alcohol and ether, using ungreased taps in the separators, and when aqueous caustic soda was required this was always carefully filtered lest traces of paraffin waxes used by the dealers for sealing the corks of the alkali bottles could have contaminated the material. The results showed a marked reduction in the amount of N.S. soluble in light petroleum but it was not eliminated completely as the following examples show.

		Part soluble in light petroleum		Part insoluble in light petroleum		Total N.S.	
	Amount of plasma (g.)	g.	% of plasma	g.	% of plasma	g.	% of plasma
1	15·49	0·0083	0·0540	0·0033	0·0210	0·0110	0·0750
2	13·76	0·0063	0·0460	0·0009	0·0060	0·0072	0·0520
3	7·69	—	—	—	—	0·0052	0·0670

A series of N.S. residues of this kind showed considerable diversity of properties. They were generally acidic in character, though not always so, but contained soaps. No. 3, for example, on ignition left an alkaline ash in minute quantity.

Thus we see that a non-sterol substance is always obtained, even after extreme precautions are used, in the analysis of plasma to the extent of 0·05–0·07 % of the plasma, and that this material consists of neutral sodium salts or acid sodium salts of fatty acids.

In order to determine if these substances were produced in our analytical procedure from the plasma-lipin material we made the following experiments, using the same precautions as regards the purity of all reagents and the complete absence of all added grease.

(*a*) 1·0062 g. of a commercial specimen of cholesterol oleate, which on heating softened at 40°, M.P. 49°, was saponified and yielded 0·057 g. of N.S., of which 0·0539 g. was soluble in light petroleum. 0·0536 g. of this was dissolved in standard alcoholic potash and titrated with standard acid. It was equivalent to 6·0 ml. of $N/50$ acid. The equivalent weight of the N.S. was thus 447 which corresponds closely to a formula of $(C_{18}H_{34}O_2)_5C_{18}H_{35}O_2Na$ or a mixture of free acid and soap in such proportion, the equivalent weight of which is 434.

(*b*) 1 ml. of ethyl oleate was treated in the same manner. The N.S. was wholly soluble in light petroleum; weight 0·0091 g. It was dissolved in alcoholic potash and corresponded in titration to 3·09 ml. of $N/50$ standard acid. The equivalent of the material was thus 147, which corresponds to a dibasic acid of similar molecular weight to oleic acid (mol. wt. 282), or to a polybasic acid of higher molecular weight, or a monobasic acid of half the size of the oleic acid molecule.

(*c*) 1 ml. of ethyl palmitate was similarly treated. The N.S. was dissolved in ammonia to form a colloidal frothy solution, and a silver salt was prepared. This weighed 0·0218 g. and on heating yielded 0·013 g. of silver. The mol. wt. of the acid of the silver salt (assuming it to be monobasic) was therefore 74.

(*d*) A specimen of oleic acid, which had been in stock for some years, was redistilled *in vacuo* at 2–3 mm. pressure, and was collected in three fractions at 160–164°, 164–185°, and 185° and upwards. The first fraction showed a correct titration value; of it were taken two portions, one of which was heated with sodium ethoxide in pure alcohol, the other with alcoholic potash made from pure reagents, and the N.S. was extracted in both experiments.

(1) 1·05 g. of oleic acid yielded 0·048 g. of N.S.

(2) 1·34 g. of oleic acid yielded 0·052 g. of N.S.

Neither of these specimens possessed any acidity whatever.

(*e*) 0·9998 g. of pure palmitic acid was heated with a solution of 1 g. of sodium in pure ethyl alcohol. The solution remained colourless and little or no deposit formed. The N.S. found was 0·155 g.; this was dissolved in standard alcoholic alkali and found equivalent to 1·1 ml. of $N/5$ acid. Equivalent weight of N.S. is therefore 70·4.

(*f*) 0·9992 g. of a specially pure commercial specimen of stearic acid was heated with alcoholic sodium ethoxide exactly as in the previous experiment. The solution behaved quite differently, as it became deep brown in colour after heating, presumably owing to impurities. It was then diluted with water and thoroughly shaken out with ether in the usual way. A bulky white precipitate separated out between the ethereal and aqueous layers. It was removed by filtration and was found to be insoluble in water or ether. After washing thoroughly with these solvents it was dried and weighed 1·015 g. It was then dissolved in alcohol giving a soapy solution of alkaline character; on spontaneous evaporation it left a dry crystalline powder. Of this 0·2995 g. was ignited and the sodium present estimated as sulphate, of which 0·0611 g. was obtained. Hence this substance contained 6·61 % of sodium. This would correspond to the formula

$$C_{18}H_{36}O_2(C_{18}H_{35}O_2Na)_6,$$

which requires Na 6·5 %.

The ether-soluble matter, the N.S., weighed 0·073 g. and was practically all soluble in light-petroleum. The portion thus soluble weighed 0·0714 g.; it was dissolved in alcoholic NaOH and found by titration to be equivalent to 2·21 ml. of $N/5$ acid, yielding an equivalent weight of 158·5.

Thus we see that, after all saponifications of fatty acids or esters thereof under these conditions, unsaponifiable matter is produced and can be extracted. Such material is almost entirely soluble in light petroleum, is either acid or neutral in reaction, and presumably consists of acid salts of fatty acids or soaps. It seems probable that these substances arise from the partial hydrolysis of the soaps, the products of this hydrolysis passing into the ether layer during extraction. This appears to agree with the results of Lester Smith [1928]. Some of the equivalent weights of these acid bodies which we determined are very low, but we do not put sufficient reliance on these micro-estimations to be able to discuss these figures.

Conclusion.

There can be little doubt that the so-called N.S. (Begleitsubstanz des Cholesterins) obtained in the analysis of blood-plasma does not contain any sterol compound. The material obtained is really an artefact produced in three ways, firstly by condensation of the impurities in the reagents during saponification, secondly from the grease used in the separating funnel stopcocks, and thirdly from the hydrolysis of the soaps with production of ether-soluble substances in the extraction process.

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CCXVII. STUDIES IN THE CHOLESTEROL CONTENT OF HUMAN PLASMA.

V. THE NATURE OF THE STEROLS IN BLOOD-PLASMA.

By JOHN ADDYMAN GARDNER and HUGH GAINSBOROUGH.

From the Biochemical Laboratory, St George's Hospital, London.

(*Received July 18th, 1934.*)

It is now generally recognised that sterol is excreted in human faeces mainly in the form of coprosterol, and that this is accompanied by smaller quantities of dihydrocholesterol (β-cholestanol) and cholesterol. It was shown long ago by Böhm [1911] and in recent years confirmed by Schönheimer *et al.* [1930], in a series of elegant experiments, that the dihydrocholesterol is excreted by the wall of the large intestine. Schönheimer *et al.* [1930] have further demonstrated the presence of dihydrocholesterol to the extent of as much as 10 % in gall-stone cholesterol; and they also claimed that they had found it in smaller proportion in the cholesterol isolated from various tissues. It is generally assumed that coprosterol is produced by bacterial reduction of cholesterol in the gut, though the two substances are not in the same stereochemical series. Schönheimer *et al.* [1930] have further convincingly shown that both dihydrocholesterol and coprosterol, when administered in food, are not absorbed from the gut into the chyle.

Another sterol, allocholesterol, an isomeride of cholesterol, has been considered as associated with sterol metabolism, firstly because its catalytic reduction *in vitro* gives rise to coprosterol, secondly because Enderlen *et al.* [1928] have shown that it is the only sterol, other than cholesterol, which when fed to an animal leads to any noteworthy quantitative increase in bile acid production.

From these observations it seemed to us desirable to investigate more closely the blood-sterol for evidence of the presence of any of these substances.

In our routine analytical work we had preserved the cholesterol digitonides obtained and these were kept in separate batches according to their origin, whether from free or ester cholesterol, or from plasma, red blood corpuscles or whole blood, from normal or diseased individuals or from pregnant women. The cholesterol from each of these digitonides was recovered by the xylene method of Windaus, and each was submitted to an elaborate process of fractional crystallisation from alcohol. The melting-points of the initial fractions from different sources were 148–149°, subsequent fractions melting at 147–148°, with no appreciable progressive lowering of the melting-point during the fractionation. The residual material in the mother-liquor was small in quantity and dried up to a transparent glassy residue, which still gave the Burchardt and Liebermann colour test, and from which some further very small quantity of cholesterol could be obtained by precipitation with digitonin. The bulk of this residual

material was found to be a resin contained in the commercial rectified xylene which we had used. This was shown by a blank experiment in which the digitonide was omitted—the resin thus obtained yielded an atypical colour change with the Burchardt-Liebermann test. In subsequent experiments, using pure sulphur-free xylene, the amount of the resinous material was almost negligible.

Micro-polarimetric estimations were made of the specific rotation of the various fractions and the results varied between $-37{\cdot}5$ and $-41{\cdot}5°$. These however being micro-estimations we are not prepared to say how far these variations were due to errors of experiment. The constancy of the melting-point in the successive fractions during crystallisation, and the high negative specific rotatory power make it very unlikely that any appreciable quantity of positively rotatory dihydrocholesterol could be present. The unfortunate contamination of our tail fractions with the xylene resin made it impossible to say that no other sterol was present. We therefore collected large quantities of human blood, mainly from hyperpietic patients. The plasma-cholesterol, free and ester separately, was isolated from these by Fex's method combined with the use of digitonin; cholesterol from the erythrocytes was also obtained in a similar manner. The digitonides thus obtained were decomposed with pure sulphur-free xylene, and the sterols obtained were fractionally crystallised. The tail portions were glassy in character and contained very little xylene resin. The final fractions of sterol were obtained as before by reprecipitation from the glassy mass with digitonin. In this way practically the whole of the sterol present was recovered, and no other sterol than cholesterol could be isolated.

About this time Schönheimer *et al.* [1930] reported on a method of estimating β-cholestanol in the presence of cholesterol based on the fact that cholesterol dibromide, left in solution after bromination of an alcoholic solution of cholesterol, is not precipitated by digitonin, whereas β-cholestanol forms an insoluble digitonide. In this process the alcoholic solution of cholesterol was brominated quantitatively by addition of an alcoholic solution of bromine; the crystalline dibromide which separated after standing in the cold was filtered off and the filtrate treated with an alcoholic solution of digitonin in the usual way; any saturated sterol precipitable by digitonin separated out in 48 hours. This method of bromination seemed at first sight to present considerable advantages over bromination by the method of Windaus, *viz.* precipitation of an ethereal solution of cholesterol by a solution of bromine in glacial acetic acid, because the latter involved the extraction of any dihydrocholesterol that was present, before the precipitation with digitonin—a method used by one of us [Gardner, 1921] in the separation of faecal sterols. In Schönheimer's method, after separation of the cholesterol dibromide, the filtrate is treated with digitonin and allowed to stand in the dark, when the precipitation of β-cholestanol digitonide takes place within 48 hours, and if it is present in quantity it begins to precipitate very quickly.

We made a series of experiments using cholesterol from human blood obtained by venesection, performed mainly for hyperpiesia and right heart failure. The cholesterol used was from total cholesterol of plasma, red blood cells and from whole blood, and in each experiment 2–4 g. were used. In none of these experiments did we obtain the slightest precipitation of a digitonide in 48 hours, and in some cases even in 3 to 4 days. If therefore this method is strictly reliable β-cholestanol was not present in the sterols we had isolated from blood. This agrees with the fractional crystallisation experiments and polarimetric determinations quoted above. On the other hand when the liquid was allowed to

concentrate spontaneously in the dark precipitates gradually appeared. These were filtered off and the filtrates allowed to concentrate further. Precipitation continued during the course of weeks and so further crops were obtained. These precipitates were separated, washed with a little alcohol and much ether, and in some cases were warmed for a few minutes with hot xylene to take out any cholesterol dibromide which might have crystallised out along with the precipitate during concentration. The precipitates were then decomposed with xylene vapour in the usual manner and the product, which still contained some bromo-derivative, was purified by crystallisation, pure cholesterol being obtained, recognised by crystalline form, melting-point and properties of the acetate. The presence of this cholesterol must have been due either to the formation of the dibromide being incomplete in alcoholic solution, even though some excess of bromine had been added, or to the cholesterol dibromide remaining in the alcoholic solution being debrominated by the action of the alcohol during the spontaneous evaporation. This latter explanation of the formation of cholesterol from its dibromide is difficult to account for chemically but it recalls the finding of Windaus and Lüders [1920] as regards the debromination of cholesterol dibromide by an alcoholic solution of sodium acetate. Nevertheless some such action seemed probable because the solution, as it became more concentrated, became more acid and contained increasing quantities of ionic bromine. In order to test this we did the following experiments:

A sample of cholesterol dibromide, M.P. 112°, prepared by bromination in alcohol, was heated in alcoholic solution on an electric bath at about 40°. After 19 hours, aliquot portions were titrated with $N/10$ NaOH and also with silver nitrate. It was found that hydrobromic acid was present to the extent of about 75 % of the theoretical value if the total bromine had been removed as HBr. 3 hours' further heating produced no further change. To the main solution chalk was added to neutralise the HBr, the solution was then filtered, found to be neutral, and was then concentrated on a water-bath to small bulk. On standing, some crystalline matter separated mixed with a brown oil. The crystalline matter was recrystallised twice from methyl alcohol and finally from ethyl alcohol. Cholesterol was obtained in typical crystalline form, M.P. 147–148°, and to the extent of 14 % of the original cholesterol used.

Another sample of cholesterol dibromide, M.P. 124°, prepared by bromination in ether and acetic acid according to the method of Windaus was treated in portions in a similar manner, some on the electric bath, and others at laboratory temperature. The heated samples showed decomposition to the same extent as before, *viz.* to about 75 % of the theoretical maximum; in the unheated samples the action was much slower, and in these specimens the liberated cholesterol was precipitated by digitonin and amounted to 2 to 3 % of the original cholesterol dibromide used, after a period of standing of 2 to 3 days.

Following this evidence of the instability of cholesterol dibromide in alcohol solution at ordinary temperatures we were led to repeat Schönheimer's experiments on gall-stone cholesterol.

5·36 g. of a specimen of cholesterol obtained from gall-stones by extraction with benzene and subsequent recrystallisation from 95 % alcohol were treated by Schönheimer's method in every detail. The cholesterol used had M.P. 148·5°, $[\alpha]_D^{17°}$ −37·4°. If we take the specific rotation of cholesterol as −41° and that of β-cholestanol as +34° the rotation observed for this specimen corresponds to a β-cholestanol content of 5 %.

The digitonide precipitate obtained in the first 48 hours after the separation of the dibromide weighed 0·9429 g. This was decomposed by xylene, and after crystallisation 0·0188 g. of recognisable β-cholestanol was obtained. It gave no Burchardt and Liebermann reaction. Subsequent crops from the crystallisation contained no recognisable β-cholestanol. Thus the amount of this

substance obtained equalled 0·35 % of the original cholesterol taken. The original liquid after removal of the first digitonide precipitate, on long standing gradually deposited further quantities of white precipitate which we examined. By treatment with xylene these were found to consist mainly of cholesterol digitonide.

We then repeated this experiment with 24·0 g. of cholesterol obtained in similar manner from mixed gall-stones of various types. This specimen melted at 145–146° and had $[\alpha]_D^{19^\circ}$ −35·6°, corresponding to a β-cholestanol content of 7·2 %. This was treated in alcohol with alcoholic solution of bromine, and the dibromide which separated weighed 21·0 g.; hence at least 12 g. of cholesterol dibromide remained in solution in the 2½ litres of alcohol used. Digitonin was added in amount sufficient to correspond with Schönheimer's figures of up to 10 % of β-cholestanol in gall-stone cholesterol. The precipitate obtained in 48 hours weighed 1·1162 g., and the filtrate therefrom was allowed to stand in open beakers in the dark for further periods, and subsequent crops of precipitate on the 5th, 19th and 33rd days were separately filtered off and examined. The initial precipitate, supposed to be β-cholestanol digitonide, was decomposed by xylene and the residue tested to show that decomposition was complete. The sterol, after removal of the xylene, was fractionally crystallised from alcohol, and the first crop weighed 0·1029 g. This gave only a trace of colour with the Burchardt and Liebermann test and had a specific rotation of −4·7°. Evidently the material was β-cholestanol but, reckoning from the rotation, contained about 30 % of cholesterol. It was then brominated by the Windaus method, a slight precipitate which formed was filtered off, the trace of bromine left was removed by sulphur dioxide and the recovered β-cholestanol after recrystallisation from alcohol melted correctly at 142–143° and gave no B. and L. reaction even on standing. We could not isolate any more β-cholestanol from subsequent crops of the first deposit.

The later deposits from the brominated solution, on further examination, yielded white crystalline materials with specific rotations of −36° to −42°, M.P. 130° to 148°, which gave typical cholesterol dibromide on bromination.

If we reckon the whole of the first crop of sterol recovered from the first 48 hours' digitonide precipitate as β-cholestanol then only 0·4 % of β-cholestanol was isolated from the original gall-stone cholesterol.

We can therefore confirm Schönheimer's finding of β-cholestanol in gall-stone cholesterol, though in our specimens the actual identifiable quantity was less than in his specimens. Nevertheless from our experience the method is of qualitative value only, as the digitonide obtained in the first 48 hours is not entirely that of β-cholestanol but contains cholesterol digitonide and possibly some digitonin if this is added in excess. We think the method is unreliable for the examination of small quantities of material, especially as we have shown above that cholesterol dibromide in alcoholic solution is unstable at ordinary temperatures. It seems to us unfair to assume, in the examination of sterols in small quantities of tissues, that any small precipitate of digitonide obtained by Schönheimer's method in 48 hours represents β-cholestanol unless this substance is further specifically identified.

We also made a series of experiments in an endeavour to detect whether any allocholesterol is present in human blood. It seemed possible that by using large quantities of fresh blood, rapidly drying by the addition of plaster of Paris and extracting with ether alone, we might be enabled to extract any allocholesterol largely unchanged along with the other ether-soluble substances. We hoped that by taking the dry ether solution we might be able to reduce any allocholesterol in this material in the cold with platinum black, according to the method of Willstätter and Meyer [1908] to the stable coprosterol, the cholesterol present being reduced to β-cholestanol. The experiments were not successful, as we were unable to get complete reduction, probably as a result of some substance in the ether extract interfering with the activity of the catalyst. We isolated cholesterol and some β-cholestanol from the partially reduced material but could not find

any coprosterol. Unfortunately the ether extract of the dried blood was slightly acid and this probably also militated against a successful result by this method. We hope to return to this investigation shortly.

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CCXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XL. THE CRYSTALLINE PIGMENTS OF SPECIES IN THE *ASPERGILLUS GLAUCUS* SERIES.

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The species in the *Aspergillus glaucus* series constitute the most common group of all the *Aspergilli* and are characterised by quite definite morphological and biochemical characters. According to Thom and Church [1926, p. 102] the general morphological characters are: "Heads green; stalks smooth (not rough or pitted) septate (articulate); sterigmata 1 series; conidia predominantly elliptical, more than 5μ in long axis (with few exceptions), mostly with roughly pitted wall, rarely smooth; most races or species producing sessile, globose, cleistocarpic yellow to orange perithecia with membranaceous walls, borne above the surface of the substrata and producing disk-shaped ascospores, colourless or nearly so."

The conidial heads are always some shade of green, but the green colour is often masked by the abundant growth of coloured perithecia or by the production of red incrustations on the aerial hyphae by certain species in the series. Macroscopically the colony colour varies widely from brilliant yellow through red to deep chocolate-brown. Blochwitz [1929] indeed divided the series into 4 pigment groups based on the macroscopic appearance of the colony, *viz. Aspergillus glaucus*, myc. *aureo*, myc. *ferrugineo*, myc. *rubro*, myc. *violaceo*.

Biochemically, the *A. glaucus* series of species forms a distinct group among the *Aspergilli*. Because of their ability to tolerate extremely high osmotic concentrations the organisms are often found on what would generally be called dry matter. They are a common cause of "mildew" in textiles. They tolerate extremely high concentrations of glucose (50 %). The optimum hydrogen ion concentration for growth is in the vicinity of neutrality with little or no growth above p_H 8 and below p_H 4. The optimum temperature range for growth is between 18 and 27° with growth most luxuriant for most species at 24°. Raistrick *et al.* [1931] carried out studies on the biochemistry of various species in the series by the "carbon balance sheet method" and concluded after an analysis of the various metabolic products that, "The *Aspergillus glaucus* group is of particular biochemical interest since the species included in it have a well marked type of metabolism...."

As early as 1897, Meissner reported that the red-orange coloration produced in the mycelium of cultures of a new unnamed species in the *A. glaucus* series was due to the formation of a coloured substance separating from the surface of the mycelial filaments. The later researches of Mangin [1909] led him to conclude that the colouring matters produced by a large number of species in the series

are characteristic. Bainier and Sartory [1911; 1912] emphasised the production of red and yellow colouring matters as diagnostic characters for various species in this series. In these early attempts to isolate the pigments no crystalline products were obtained, the conclusion that the pigments were the same in the various species being based solely on the colour reactions of a mycelial extract. Research along these lines has been sporadic in recent years. Blochwitz [1929] attempted to use the solubilities of the various colouring matters present as a means of separation and claims that there are four mycelial pigments—yellow, red, brown and violet—none of which was, however, obtained crystalline or in a pure condition.

In the present research a successful attempt has been made to isolate these colouring matters, so apparently characteristic of the *A. glaucus* series, in the crystalline state and to determine whether what has hitherto been regarded as a preliminary diagnostic and confirmative character may not actually be an absolute character either of separate species or of the group as a whole.

Three crystalline pigments have been isolated by solvent extraction of the dried mould substance of 25 species of the *A. glaucus* series:

(1) A yellow pigment, which we propose to call *flavoglaucin*, has the empirical formula $C_{19}H_{28}O_3$, is probably derived from the perithecia and is readily soluble in light petroleum. It is elaborated by all 25 species and is therefore series-specific.

(2) An orange pigment, which we have named *auroglaucin*, is less soluble in light petroleum and has the empirical formula $C_{19}H_{22}O_3$. This pigment is elaborated by 15 of the species examined, and these organisms therefore form a definite sub-group in the series.

(3) A red pigment, which we have termed *rubroglaucin*, is least soluble in light petroleum, has the formula $C_{16}H_{12}O_5$ and displays the characteristics of a polyhydroxyanthraquinone. It has been isolated accompanied by flavoglaucin from 4 species.

Two species which produce flavoglaucin, auroglaucin and a second red pigment distinct from rubroglaucin form a transition group between the two groups previously described. Certain other species give rise to dark-coloured pigments which have not yet been obtained crystalline or in a pure condition.

To prove the specificity of flavoglaucin, auroglaucin and rubroglaucin for the species of the *A. glaucus* series, 19 different species, representing practically all the groups in the genus *Aspergillus*, were investigated, and in no case could any of the *glaucin* pigments be isolated or detected.

In order to determine the effect on pigment production of variations in the period and temperature of incubation, p_H and nitrogen and carbon sources, a number of experiments were carried out and are discussed in a later section of this paper. As a result of these experiments a medium has been found on which markedly increased growth and pigment production are obtained, and large quantities of flavoglaucin (191 g.) and auroglaucin (65 g.) have been isolated for future work on the molecular constitution of these pigments.

Experimental.

Species of the Aspergillus glaucus *series used.*

For the purpose of this research a large number of species in the series was collected, so that the study could be made as comprehensive as possible. The 25 species investigated represent practically all known in culture, or identifiable

from previous descriptions, as well as a number of newly isolated species. To Prof. Abbé Biourge of the University of Louvain we are grateful for the following species: *A. profusus* (Bainier) Hann. (London School of Hygiene and Tropical Medicine Catalogue, No. A 27); *A. oriolus* Biourge (A 30); *A. lovaniensis* Biourge (A 31); *A. Dierckxii* Biourge (A 33); *A. argillaceus* Biourge (A 34); *A. mongolicus* Biourge (A 36); *A. fumigatoides* Bainier and Sartory (A 38); *A. echinulatus* (Delacroix) Thom and Church (A 28); and *A. albidus* Spegazzini (A 35). We are grateful to Dr Charles Thom, U.S.A. Department of Agriculture, for a culture of *A. mutabilis* Bainier and Sartory (A 40). From the Centraalbureau voor Schimmelcultures at Baarn, Holland, we have purchased the following well-defined species: *A. glaucus* mut. *alba* Blochwitz (A 16); *A. repandus* Bainier and Sartory (A 17); *A. umbrosus* Bainier and Sartory (A 18); *A. pseudoglaucus* Blochwitz (A 19); *A. glaucus* Link (Ac 36); *A. ferrugineus* Fuckel (Ac 39); *A. mollis* Bainier and Sartory (Ac 58); *A. Scheelei* Bainier and Sartory (Ac 60); and *A. disjunctus* Bainier and Sartory (Ac 61). In addition we are indebted to Mr G. Smith, one of our colleagues, for the contribution of the following species isolated by him from infected cotton: *A. ruber* (Spieckermann and Bremer) Thom and Church (No. 6); *A. repens* (Corda) Sacc. (No. 13); *A. Chevalieri* (Mangin) Thom and Church (No. 50); *A. Amstelodami* (Mangin) Thom and Church (No. 75); and *A. herbariorum* series *minor* (Mangin) Thom and Church (No. 96). *A. novus* Wehmer (Ac 46) was purchased originally in 1923 by Raistrick from Collectio microbiologica, Pribřam, Vienna, and is placed by Thom and Church [1926] in the *A. flavus-oryzae* group, but both Blochwitz [1929] on purely morphological grounds, and Raistrick *et al.* [1931] on biochemical grounds, contest the classification and place this organism in the *A. glaucus* series. We have included this species as a member of the *A. glaucus* series, and by the subsequent isolation of both flavoglaucin and auroglaucin can further substantiate the claims of both Blochwitz and Raistrick *et al.*

Species other than those of the A. glaucus *series.*

To show that the pigments isolated are specific for the *A. glaucus* organisms a group of 19 organisms representative of practically all the other types of *Aspergilli* was investigated. These cultures consisted of well-defined strains of the following organisms obtained from the stock collection of fungi of the Division of Biochemistry of the London School of Hygiene and Tropical Medicine. Where possible, strains known to produce yellow, orange or red pigmented growths were employed. The organisms were: *A. fumigatus* Fresenius (No. 47); *A. versicolor* (Vuillemin) Tiraboschi (No. 35 and A 14); *A. ustus* (Bainier) Thom and Church (Ac 84); *A. nidulans* (Eidam) Winter (No. 79); *A. flavipes* (Bainier and Sartory) Thom and Church (No. 89); *A. Wentii* Wehmer (Nos. 2 and 41); *A. ochraceus* Wilhelm (Nos. 69 and 92); *A. auricomus* (Guéguen) (A 41); *A. vitellinus* (Ridley) (A 42); *A. ostianus* Wehmer (Ac 35); *A. terricola* Marchal (Ac 22); *A. tamarii* Kita (No. 72); *A. oryzae* (Ahlburg) Cohn (A 11); *A. flavus* Link (A 12); *A. terreus* Thom (Nos. 3 and 37).

All the cultures used in the investigation were kept on wort agar slopes and before use in the experiments were plated out; single colony isolations were made, and these were used for growing pure cultures for subsequent inoculations.

Preliminary growth experiments on synthetic media.

The species in the *A. glaucus* series are characterised by extremely poor growth on the usual synthetic media but grow luxuriantly on natural media. A series of experiments was therefore carried out to determine the rate of growth

on various synthetic media. For this purpose each of the following six nitrogen sources: sodium nitrate, ammonium sulphate, ammonium nitrate, asparagine, ammonium tartrate and peptone was employed with each of the following five carbon sources: glucose, maltose, sucrose, glycerol and soluble starch.

A quantity of the usual Czapek-Dox medium was made up without carbon or nitrogen source, having the following composition: KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; distilled water, 1 litre. 50·0 g. of the carbon source and an amount of nitrogen source equivalent in nitrogen to 2·0 g. $NaNO_3$ were added, and the media were solidified by the use of 2 % agar-agar. Plates were poured and single colony inoculations of the five test organisms, *A. Chevalieri* (No. 50); *A. Amstelodami* (No. 75); *A. repens* (No. 13); *A. ruber* (No. 6); and *A. herbariorum* (No. 96) were made. The plates were incubated at 24° and observed daily for rate of growth, as determined by measuring the colony, and for pigment production. It was found that all the sources of carbon used except soluble starch were quite comparable. The combination of glucose and diammonium tartrate gave a growth which was highly pigmented, and whilst not as spreading as some of the others was far more dense. On the glucose-peptone medium growth was abundant but floccose and consisted of an abundance of conidia with few perithecia. Pigmentation on the sodium nitrate and on the asparagine media was much less marked. The ammonium sulphate and ammonium nitrate media produced very poor growths which consisted mainly of conidia. The medium chosen, therefore, for the cultivation of the *A. glaucus* species on a large scale was a modified Czapek-Dox medium containing 5 % glucose as a source of carbon and 0·216 % diammonium tartrate as a source of nitrogen.

Growth of the organisms and isolation of the crude pigments.

For each organism investigated a modified Czapek-Dox medium was made up of the following composition: glucose, 1750 g.; KH_2PO_4, 35·0 g.; KCl, 17·5 g.; $MgSO_4, 7H_2O$, 17·5 g.; $FeSO_4, 7H_2O$, 0·35 g.; distilled water, 34 litres. 340 ml. quantities were distributed in each of 100 one litre conical flasks and sterilised by steaming on 3 successive days. A solution containing 75·6 g. diammonium tartrate per litre was sterilised at the same time and after sterilisation of the separate solutions 10 ml. of the diammonium tartrate solution were added, aseptically, to each of the flasks. This precaution was taken to prevent discoloration of the medium, which occurs when the diammonium tartrate is sterilised along with the rest of the medium. Each flask was then sown with a spore suspension of the chosen organism grown on wort agar slopes. Usually 4 flasks were sown from each slope. The flasks were then incubated at 24° until no further apparent decrease in the sugar concentration took place, usually from 4–8 weeks. At the end of this period the mycelium (including perithecia and conidia), which with different species varied in colour from a pure yellow or green to a red-brown, was filtered off, washed carefully with cold distilled water, pressed and dried in a vacuum-oven at 35–40°. The dried mycelium was ground to a fine powder in a coffee-mill, mixed with ether-extracted pumice and exhaustively extracted with light petroleum (B.P. 40–50°) in a Soxhlet apparatus. Light petroleum (B.P. 40–50°) was, in general, found to be the most suitable solvent for the extraction of the pigments, since the crude product contains fewer impurities than after extraction with ether, methyl alcohol, chloroform or acetone. The pigments are more readily soluble in these latter solvents and in the case of organisms of Group I, which will be discussed later, ether serves as the most efficient solvent. Using light petroleum (B.P. 40–50°) the extraction

time varies from 1–2 working days with those organisms producing flavoglaucin and auroglaucin only, to 3–5 working days for those species which produce these pigments together with rubroglaucin or the unnamed red pigment. The total yields of crude pigments varied from 1·27 g. per 100 flasks with *A. Dierckxii* to 23·80 g. per 100 flasks with *A. mongolicus*. The yield varied from 1·69 % to 15·6 % of the weight of the dry mycelium produced.

Composition of the crude pigments and isolation from them of the pure pigments.

A. Group I.

In this group are included 13 species which produce a crude pigment consisting entirely of a mixture of flavoglaucin and auroglaucin. The yields and characteristics of these pigments are given in Table I.

Table I. *Organisms of Group I.* (*Producing flavoglaucin and auroglaucin.*)

Organism		No. of flasks	Incubation period days	Dry mycelium per flask g.	Total crude pigment mixture g.	Total crude flavoglaucin g.	M.P.* of flavoglaucin ° C.	Total crude auroglaucin g.	M.P.* of auroglaucin ° C.
Aspergillus Chevalieri	(No. 50)	98	24	0·839	2·52	2·42	105	0·100	151
„ *Amstelodami*	(No. 75)	99	28	1·010	2·75	2·65	103–105	0·100	150–151
„ *novus*	(Ac 46)	98	36	1·280	15·20	11·815	102–103	3·385	152
„ *repens*	(No. 13)	98	40	0·928	4·18	3·285	102–103	0·900	152
„ *glaucus*, Link	(Ac 36)	98	33	1·225	9·47	7·80	101–103	1·670	151
„ *Scheelei*	(Ac 60)	96	39	1·031	8·10	7·525	99–102	0·575	151–151·5
„ *mollis*	(Ac 58)	98	31	1·306	6·11	5·78	98–100	0·330	151–152
„ *argillaceus*	(A 34)	99	48	0·879	5·63	4·72	99–101	0·910	148–150
„ *Dierckxii*	(A 33)	95	41	0·789	1·27	1·175	97	0·095	147–149
„ *profusus*	(A 27)	98	48	1·104	5·41	4·760	101	0·650	148
„ *fumigatoides*	(A 38)	97	32	1·010	5·05	4·20	100	0·850	149·5–151
„ *disjunctus*	(Ac 61)	99	42	1·032	3·02	2·28	102	0·740	151
„ *pseudoglaucus*	(A 19)	99	39	1·025	2·67	2·49	105	0·180	146

* Mixed melting-points not depressed on admixture with pure sample.

An average sample of the crude pigment sintered at 85–90° and melted indefinitely from 120° upwards. Its solubilities in various solvents showed that it consisted of flavoglaucin which is insoluble in water, sparingly soluble in cold but moderately soluble in hot light petroleum, and freely soluble in cold ether, methyl alcohol, ethyl alcohol, chloroform, acetone, benzene and ethyl acetate; and auroglaucin which is almost insoluble in cold light petroleum, slightly soluble in cold, but readily in hot, methyl or ethyl alcohol, freely soluble in ether, acetone or ethyl acetate.

In order to obtain pure samples of flavoglaucin and auroglaucin the difference in their solubilities in light petroleum was first utilised as a means of preliminary separation. For example, 2·75 g. of the crude pigment mixture from *A. Amstelodami* were suspended in 85 ml. hot (B.P. 50–60°) light petroleum and boiled. The greenish yellow solution was filtered from the orange residue (0·10 g.). The first crop of crystals consisted of 0·65 g. of brilliant yellow feathery needles, M.P. 95–97°. These were recrystallised twice from 90 ml. and 50 ml. (B.P. 50–60°) light petroleum yielding 0·215 g. of lemon-yellow long feathery needles of flavoglaucin, M.P. 105° (sharp, without decomposition), which was not altered by further recrystallisation.

The crude orange pigment remaining after boiling with light petroleum was recrystallised from hot methyl alcohol. 0·77 g. of crude material from *A. disjunctus* (sinters at 88°, melts indefinitely from 120° upwards) was crystallised from 50 ml. methyl alcohol. The first crop consisted of 0·65 g. of slender needles melting at 143–144°; second crop, 0·09 g., M.P. 146°. Recrystallisation of the first crop from 40 ml. methyl alcohol yielded 0·40 g. of slender orange hairy needles of auroglaucin, M.P. 152° (sharp, without decomposition), which after repeated crystallisation from methyl alcohol still melted at 152°.

It was often found extremely difficult to separate the last traces of auroglaucin from the flavoglaucin and to crystallise the flavoglaucin by the method described above. The following procedure was therefore utilised to effect complete separation. 2·27 g. of a crude mixture of flavoglaucin and auroglaucin (M.P. sinters 85–90°, melts indefinitely at 120° upwards) was dissolved in 25 ml. of hot methyl alcohol. The first crop of crystals obtained consisted of 0·65 g. of auroglaucin, M.P. 148°. The mother-liquor was cooled in ice-water and a second crop consisting of 0·055 g., M.P. 142–144°, was collected. Distilled water was added until the mother-liquor was turbid, and the solution was warmed until the precipitate dissolved. The process was repeated. The residual auroglaucin separated as a golden oil. When no further oil separated out the aqueous methyl alcohol solution was allowed to cool slowly, the flavoglaucin separating out on standing.

Analysis and properties of flavoglaucin. Flavoglaucin crystallises in lemon-yellow, long feathery needles and melts at 105° without decomposition. Found: C, 74·87, 75·07; H, 9·00, 8·91%; mol. wt. (cryoscopic in camphor), 299, 306. Contains no methoxyl or nitrogen. $C_{19}H_{28}O_3$ requires C, 75·00; H, 9·22%; mol. wt. 304. (Analyses by Schoeller, Berlin.) It dissolves sparingly in cold, and moderately in hot, light petroleum to give a brilliant yellow-green solution. It dissolves readily in ether, acetone, methyl and ethyl alcohols, glacial acetic acid and ethyl acetate. It is insoluble in hot or cold water but moderately soluble in hot 80 % aqueous methyl or ethyl alcohol.

It gives the following colour reactions: With cold concentrated sulphuric acid a beautiful intense red colour is produced which blackens on heating. The solid is not immediately soluble in cold aqueous NaOH but dissolves slowly to give an orange-coloured solution which fades to pale yellow.

With cold concentrated nitric acid the solid dissolves slowly to give an orange solution which is unchanged on heating. If the nitric acid solution is treated with aqueous NaOH a yellow solution is obtained which turns orange on the addition of an excess of NaOH.

An alcoholic solution of flavoglaucin was used for the following tests:

(*a*) The addition of an aqueous solution of NaOH gives an orange-red colour which rapidly fades to yellow; sodium carbonate gives a transient pale orange; $NaHCO_3$ gives a faint orange and borax solution no coloration. Ammonia (concentrated) gives a deep red colour which fades slowly to orange-brown.

(*b*) Aqueous ferric chloride gives only a faint yellow-orange colour.

(*c*) No coloration is produced upon the addition of aqueous solutions of nickel sulphate, potassium alum or magnesium chloride.

(*d*) Dilute aqueous permanganate solution is decolorised, more slowly than with an alcoholic solution of auroglaucin.

(*e*) With 2:4-dinitrophenylhydrazine in aqueous HCl a red precipitate is immediately produced.

Analysis and properties of auroglaucin. Auroglaucin crystallises in slender orange needles, M.P. 152°, without decomposition. (Found: C, 76·52, 76·40;

H, 7·57, 7·43 %; mol. wt. (cryoscopic in camphor), 297, 298; contains no methoxyl or nitrogen. $C_{19}H_{22}O_3$ requires C, 76·50; H, 7·44 %; mol. wt. 298.) It dissolves very sparingly in cold and sparingly in hot light petroleum. It is easily soluble in ether and acetone, sparingly soluble in cold and moderately soluble in hot methyl or ethyl alcohol to give a yellow solution. It is insoluble in hot or cold water.

The solid gives the following colour reactions: With concentrated sulphuric acid in the cold auroglaucin is charred. It is not immediately soluble in cold aqueous NaOH but dissolves slowly to give an orange-red solution. With concentrated nitric acid the solid dissolves slowly to give an orange-brown solution which turns yellow on warming and upon the addition of NaOH turns pale straw-yellow.

A dilute alcoholic solution of auroglaucin which is brilliant yellow in colour gives the following colour reactions:

(*a*) With aqueous NaOH a deep red coloration with a tinge of violet is produced which fades to an orange-brown on standing. Ammonia (concentrated) gives an intense violet which fades slowly; dilute ammonia (2*N*) gives a transient violet. Sodium carbonate gives an orange solution which turns red-violet on standing; $NaHCO_3$ gives a pale yellow colour which turns pink and then fades. Aqueous borax solution produces an orange colour which turns to brilliant red-violet on standing and then slowly fades to pale brown.

(*b*) Aqueous ferric chloride solution gives an orange-brown colour.

(*c*) No colour is produced by aqueous solutions of nickel sulphate, magnesium chloride or potassium alum.

(*d*) No precipitate is formed on the addition of an aqueous HCl solution of 2:4-dinitrophenylhydrazine.

(*e*) A dilute aqueous solution of permanganate is rapidly decolorised.

Further extraction of the mycelia of Group I organisms. The mycelia of the 13 organisms of Group I after exhaustive extraction with light petroleum in the process of extracting flavoglaucin and auroglaucin were then extracted successively with ether, acetone, chloroform, methyl alcohol, ethyl alcohol and ethyl acetate, but in no case could any further pigment extracts be obtained. These organisms constitute therefore a definite biochemical sub-group in the *A. glaucus* series characterised by the presence of only the two pigments—flavoglaucin and auroglaucin.

B. Group II.

A. oriolus (A 30) and *A. albidus* (A 35) are unique among the organisms investigated in that they alone produce in perceptible amounts, in addition to flavoglaucin and auroglaucin, an unnamed red pigment which has not yet been obtained in a pure condition.

A batch of 100 flasks of *A. oriolus* was sown on the modified Czapek-Dox medium given on p. 1643. The flasks were incubated at 24° for 29 days. The weight of washed, vacuum-dried mycelium was 132·5 g. The mycelium was ground in a coffee-mill and extracted with light petroleum for approximately 24 hours. The yield of crude pigment thus obtained was 13·53 g., corresponding to 10·2 % of the mycelium formed or 2·42% of the sugar metabolised. The crude mixture was suspended in 100 ml. of cold methyl alcohol and filtered from the red insoluble residue which consisted of 0·60 g. of a pale red product, obviously a mixture of red and colourless material. These two substances have not been satisfactorily separated owing to their similar solubilities in all solvents. The methyl alcohol solution was evaporated to about 20 ml. at atmospheric pressure and allowed to

cool slowly. The first crop of crystals consisted of 0·13 g. of orange needles, M.P. 143–145°. Recrystallisation from 7 ml. of methyl alcohol gave 0·075 g. of feathery needles, M.P. 150–151°, which on admixture with pure auroglaucin (M.P. 152°) melted at 150–152°. Auroglaucin is thus one of the constituents of the crude pigment of *A. oriolus*. The methyl alcohol mother-liquors from the first crop of auroglaucin were evaporated to dryness and the residual flavoglaucin was crystallised from light petroleum, yield about 12 g.

A. albidus was treated in a similar fashion. From 97 flasks incubated for 43 days, 123·2 g. of mycelium were obtained which yielded 7·45 g. of crude pigment, from which 7·24 g. of flavoglaucin and 0·21 g. of auroglaucin were isolated. Owing to the small amount present, the red pigment could not be separated but its presence was detected by the formation of characteristic lakes with aluminium and magnesium oxides.

Unlike the organisms of Group I, the mycelia of *A. oriolus* and *A. albidus*, after exhaustive extraction with light petroleum, still retain their original red-orange colour, and upon further extraction with either methyl alcohol, ethyl alcohol, chloroform or acetone give a deep cherry-red extract which upon evaporation gives a black amorphous material which has evaded all attempts at crystallisation.

These two organisms therefore constitute a second group in the pigment classification of the *A. glaucus* series, forming a transition group between groups I and III.

C. Group III.

The remaining ten organisms investigated fall into a group characterised by the presence of at least one red pigment soluble in light petroleum associated with the presence of flavoglaucin and the absence of auroglaucin. The yields and characteristics of these pigments are given in Table II.

Table II. *Organisms of Group III.* (*Producing flavoglaucin and a red pigment.*)

Organisms		No. of flasks	Incubation period days	Mycelium per flask g.	Total crude pigment mixture g.	Total crude flavoglaucin g.	M.P.* of flavoglaucin °C.	Total red pigment g.	M.P.* of rubroglaucin °C.
Aspergillus ruber	(No. 6)	97	28	0·878	4·200	3·950	99–100	0·250	172–173
„ *ferrugineus*†	(Ac 39)	95	51	0·958	3·340	3·280	97	Trace	173–174
„ *glaucus* mut. *alba*	(A 16)	91	51	1·412	20·050	18·370	104	1·680	173–175
„ *umbrosus*	(A 18)	96	41	1·860	5·120	Trace	102	Trace	170–172
„ *mongolicus*	(A 36)	99	57	2·120	23·800	21·700	103–105	2·100	Mixed M.P. 167–170
„ *echinulatus*	(A 28)	100	52	0·880	1·240	0·570	95–96	0·670	—
„ *lovaniensis*	(A 31)	93	62	1·230	7·690	7·430	99–100	0·260	—
„ *herbariorum*	(No. 96)	100	56	1·360	1·750	0·750	98	1·00	—
„ *mutabilis*	(A 40)	98	33	1·620	3·920	0·870	104–105	3·050	—
„ *repandus*†	(A 17)	98	58	0·809	3·000	2·940	96	0·060	—

* Mixed melting-point not depressed on admixture with authentic flavoglaucin or rubroglaucin.
† Produces a red pigment, M.P. 193–195°.

The red pigment of *A. ruber* (No. 6), *A. ferrugineus* (Ac 39), *A. glaucus*, mut. *alba* (A 16) and probably *A. umbrosus* (A 18) has been isolated in the pure crystalline condition and has been named *rubroglaucin*.

In the case of *A. umbrosus*, as with most of the members of this group, the bulk of the light petroleum extract consists of flavoglaucin, together with small

amounts of a colourless impurity and of a red pigment. By slow crystallisation of the mixture of red and colourless products of *A. umbrosus* from ethyl acetate, a mixture of almost colourless needles and ruby-red crystals was obtained. The melting-point of the mechanically separated red crystals was 170–172°, but upon admixture with a pure sample of rubroglaucin, M.P. 172–173°, the melting-point was depressed to 167–170°. This may have been due to the presence of a small amount of the colourless product.

A. ferrugineus (Ac 39) produces in addition to flavoglaucin and rubroglaucin a third pigment, also red, which differs from rubroglaucin in melting-point, 193–195°, and in colour reactions. The melting-point of a mixture of the two shows a depression. The same red pigment, as indicated by similar melting-point, non-depression of the melting-point upon admixture of the two and similarity of colour reactions, was isolated from *A. repandus* (A 17), but in this case no rubroglaucin could be detected. As was the case with *A. ferrugineus*, only a trace could be isolated and after purification there was insufficient material for analysis. It is hoped that in a later communication the isolation and properties of this second red pigment will be described.

The remaining species produce flavoglaucin and a red pigment in each case, but it has been impossible to isolate these red pigments in a state of purity, owing either to the small amounts present or to the presence of large amounts of impurities which could not be selectively removed. It is quite certain, however, that the red pigment in all these remaining organisms is not rubroglaucin since the colour reactions vary from species to species, especially the coloured lakes produced with aluminium and magnesium oxides. Group III represents therefore a general group which contains, probably, a number of sub-groups.

For the isolation of pure rubroglaucin the mycelium from 100 flasks of *A. ruber* grown on the modified Czapek-tartrate medium and amounting to 85·2 g. was extracted thoroughly with light petroleum (B.P. 40–50°) for approximately 40 hours, and the reddish yellow mixture of pigments was collected. 4·20 g. of crude material were suspended in 25 ml. methyl alcohol in which flavoglaucin is readily soluble and rubroglaucin only very sparingly soluble. The yellow solution was filtered off and the red residue, 0·25 g., M.P. 162–164°, was collected and crystallised from 20 ml. of ethyl acetate. The first crop consisted of 0·15 g. of short, blunt, ruby-red needles, M.P. 169–170°. This was then twice recrystallised from ethyl acetate and once from butyl alcohol, the melting-point remaining unchanged at 172–173°.

Analysis and properties of rubroglaucin. Rubroglaucin crystallises in short, blunt, ruby-red needles, M.P. 172–173°. (Found on material dried *in vacuo* at 60–65°: C, 67·16, 67·16; H, 4·34, 4·30 %; mol. wt. 336, 340, cryoscopic in camphor; $C_{16}H_{12}O_5$ requires C, 67·58; H, 4·25 %; mol. wt. 284·1 Schoeller, Berlin.)

Rubroglaucin is slightly soluble in methyl alcohol, ethyl alcohol and ether; moderately soluble in cold butyl alcohol, benzene, acetone and ethyl acetate, and readily soluble on heating. It is very slightly soluble in light petroleum and insoluble in hot or cold water. Its solution in organic solvents is orange-coloured with a slight green fluorescence. An alcoholic solution gives the following colour reactions:

(*a*) The addition of an aqueous solution of ammonia or sodium hydroxide produces an intense red-violet colour which is destroyed on the addition of aqueous sodium hypochlorite solution.

(*b*) The addition of aqueous nickel sulphate, magnesium chloride, potassium alum, lead acetate or borax solution produces a rose-pink colour.

(*c*) Aqueous ferric chloride solution produces a faint green colour.

(*d*) Aqueous copper sulphate solution produces a violet-pink colour.

(*e*) Upon shaking an aqueous alcoholic solution of rubroglaucin with magnesium oxide a deep blue-violet colour is produced; with calcium oxide a violet colour; with aluminium oxide a blue colour.

The solid produces an intense violet solution in concentrated H_2SO_4 and a beautiful red-violet in NaOH. It is insoluble in aqueous $NaHCO_3$ and aqueous Na_2CO_3.

Experiments with Aspergilli *other than members of the* A. glaucus *series.*

In order to show that flavoglaucin, auroglaucin and rubroglaucin are characteristic of and specific for the species in the *A. glaucus* series, an experiment was undertaken in which nineteen species of *Aspergillus* other than members of the *A. glaucus* series were examined. The organisms were selected so as to be fully representative of the entire genus *Aspergillus*, and where possible, species producing yellow to red coloured colonies were employed. The organisms, listed on p. 1642, were grown in batches of 10 flasks on the usual modified Czapek-ammonium tartrate medium as were the *A. glaucus* organisms, and under the same conditions. The mycelia were filtered off, washed, pressed, dried *in vacuo* and thoroughly extracted with light petroleum (B.P. 40–50°). Each of the extracts was treated in the same manner as with species in the *A. glaucus* series. As a further test, since in no case was any pigmented precipitate obtained on evaporating the light petroleum extract to a small volume, the evaporation was carried to dryness, and the residue, if any, was dissolved in methyl alcohol. To a portion of the methyl alcohol solution was added an aqueous HCl solution of 2:4-dinitrophenylhydrazine, which would produce a red precipitate if flavoglaucin were present. Water was added to another portion in order to precipitate any flavoglaucin or auroglaucin. None of the 19 organisms appears to produce any of the glaucin pigments, and it seems certain therefore that the pigments are specific for the species in the *A. glaucus* series.

Production of flavoglaucin and auroglaucin under varying cultural conditions.

I. *Period of incubation.*

To follow the quantitative production of flavoglaucin and auroglaucin 150 flasks, each containing 350 ml. of the usual Czapek-ammonium tartrate medium, were made up, sterilised and sown with equal amounts of a spore suspension of heavily sporing cultures of *A. novus* (Ac 46). This species was chosen since it rapidly gives a good growth, and the only pigments it produces are flavoglaucin and auroglaucin, both of which are formed in good yield. The flasks were incubated at 24° in the dark. Batches of 8 flasks in the early stages of growth and of 5 flasks in the later stages were removed periodically, and the mycelium was filtered off, washed, pressed, dried *in vacuo* and weighed. The dry mycelium was ground to a fine powder and thoroughly extracted with ether. The ether extract was evaporated to dryness and its weight determined after drying to constant weight *in vacuo* over concentrated H_2SO_4. This product is termed the *total crude extract*. This was then rubbed up with light petroleum (50–60°) and the volume made up to approximately 70 ml. of light petroleum per g. of extract. After boiling on a hot bath for 8–10 minutes the orange residue was filtered off, dried *in vacuo* and weighed. This is called *crude auro-*

glaucin. The difference between the total crude extract and the crude auroglaucin is taken as the *crude flavoglaucin*. It contains, of course, in addition to flavoglaucin a certain amount of fat and other products soluble in light petroleum.

The results of analyses carried out over a period of 75 days are given in Table III. It will be seen that growth increases until the forty-sixth day and

Table III. *Growth and pigment production by* Aspergillus novus (*Ac 46*) *on the Czapek-tartrate medium**.

Type of growth	Period of incubation days	Weight of mycelium g. per flask	Crude extract g. per flask	Crude extract per g. mycelium mg.	Crude flavoglaucin per flask g.	Crude auroglaucin per flask g.	Ratio $\frac{\text{flavoglaucin}}{\text{auroglaucin}}$
Groundwork of hyphae	5	0·074	0·0074	99·3	0·0036	0·00375	0·96
Formation of perithecia	7	0·128	0·0203	190·9	0·0155	0·005	3·1
	9	0·148	0·0365	247·5	0·030	0·0066	4·55
	11	0·189	0·064	338·3	0·052	0·012	4·33
Formation of conidia	13	0·313	0·066	211·1	0·066	Trace	—
Complete normal growth	15	0·476	0·091	192·4	0·071	0·020	3·55
	17	0·679	0·126	185·1	0·095	0·031	3·07
	19	0·862	0·155	179·4	0·110	0·044	2·50
	23	0·965	0·205	212·5	0·148	0·057	2·60
	27	1·063	0·217	204·5	0·160	0·058	2·76
	33	1·230	0·234	189·9	0·178	0·056	3·18
	35	1·287	0·255	197·9	0·185	0·070	2·64
	38	1·242	0·250	201·4	0·163	0·087	1·87
	42	1·366	0·259	189·9	0·183	0·076	2·41
	46	1·372	0·270	198·4	0·193	0·078	2·47
Period of autolysis	50	1·172	0·228	194·8	0·168	0·060	2·80
	54	1·028	0·206	200·8	0·154	0·052	2·96
	58	1·066	0·206	190·5	0·140	0·063	2·22
	65	1·093	0·212	190·4	0·153	0·059	2·59
	75	1·005	0·210	208·9	0·149	0·061	2·44

* Temperature of incubation 24° in all cases.

production of crude pigment increases correspondingly. During the first 5 days of growth when the groundwork of hyphae is being laid down, there is formed only a small percentage of crude pigment. From the fifth to eleventh days—during the period of abundant perithecia formation—the amount of pigment increases to almost 34 % of the total weight of mould substance. After this period, from the eleventh to thirteenth days, when conidia formation takes place almost exclusively, there is no additional production of pigment. From the thirteenth day onwards the percentage of pigment in the mycelium remains at a fairly constant level over the entire period of the experiment, being between 179·4 and 212·5 mg. per g. of mycelium, the average being 196·0 mg.

During the period when growth falls off (after 46 days) and there is a decrease in the weight of mould substance, due probably to autolysis, there is a corresponding decrease in the total crude extract so that the percentage amounts of pigment in the mycelium remain about the same as for the earlier figures.

During the whole period of growth there is a steady increase in the amounts of both flavoglaucin and auroglaucin, the latter being produced only very slowly and in smaller quantities. In the early stages of growth the ratio of flavoglaucin to auroglaucin rises very rapidly from about 1·0 to 4·5, but soon the ratio falls to a figure between about 2·0 and 3·0. It is interesting to note that from the eleventh to the thirteenth days, when abundant conidia formation began abruptly,

there was apparently no change in the total pigment production but practically all of the auroglaucin had disappeared. It is thus possible that auroglaucin has some function connected with conidia formation. The analytical figures seem to give very little indication as to whether the pigments are produced independently or whether one is the precursor of the other.

II. *Temperature of incubation.*

To determine the optimum temperature for pigment production by *A. novus* (Ac 46), a quantity of the usual Czapek-ammonium tartrate medium was made up and distributed in one litre flasks in 350 ml. quantities. The flasks were sterilised and sown with equal amounts of a heavy spore suspension of the organism. Batches of 5 flasks were incubated at room temperature (17–19°), 24°, 27°, 32° and 37°. After 35 days' incubation the growths were filtered off, washed, dried and weighed, ground to a fine powder and extracted, and the pigments were isolated as described on p. 1649. From the results of the experiment given in Table IV it will be seen that optimum growth as well as optimum pigment production is given at 24°. There is no growth at 32° and 37° but after 35 days at these temperatures, upon further incubation at room temperature, the spores germinated quite readily. It appears that incubation temperatures above 24° impede growth and pigment production to a greater extent than temperatures below 24°.

Table IV. *Effect of temperature on the growth and pigment production of* Aspergillus novus (*Ac 46*)*.

Temperature of incubation °C.	Weight of mycelium per flask g.	Weight of crude extract per flask g.	Crude extract per g. of mycelium mg.	Crude flavo-glaucin per flask g.	Crude auro glaucin per flask g.
17–19 (room temp.)	1·091	0·189	173·2	0·132	0·060
24	1·287	0·255	197·9	0·185	0·070
27	0·807	0·078	96·6	0·078	Trace
32	No growth	—	—	—	—
37	No growth	—	—	—	—

* Period of incubation in all cases was 35 days.

III. p_H *of the medium.*

350 ml. quantities of the usual Czapek-tartrate medium were distributed among each of 40 one litre conical flasks and sterilised. After sterilisation the appropriate amounts of sterile solutions of either *N* HCl or *N* NaOH were added to bring the p_H values to 3·0, 4·0, 6·0, 7·0, 8·0 and 9·0. The p_H of the unadjusted medium was 5·4. The flasks were sown with a heavy spore suspension of *A. novus* (Ac 46) and incubated at 24° for 35 days.

Upon analysis of growth and pigment production in the manner described previously the results given in Table V were obtained. It appears that the unadjusted medium is the best in so far as pigment production is concerned. The yield of mould substance is greater at p_H 6 and p_H 7 but the pigment yield is smaller. Below p_H 5·4 and above p_H 8 growth and pigment production are very poor. At p_H 4 the formation of perithecia is greatly impeded, the growth being predominantly conidial.

The final p_H values of the media were determined, and it was found that in every case where growth occurred the medium tended to become acid and seemed to approach a fairly constant figure of about p_H 3·2–3·5.

Table V. *Effect of hydrogen ion concentration upon the growth and pigment production of* Aspergillus novus (*Ac 46*)*.

Initial p_H	Weight of mycelium per flask g.	Weight of crude pigment extract per flask g.	Crude extract per g. mycelium mg.	Crude flavo-glaucin per flask g.	Crude auro-glaucin per flask g.	Final p_H
3·0	No growth	—	—	—	—	—
4·0	0·795	0·058	74·8	0·058	Trace	3·0
5·4	1·287	0·255	197·9	0·185	0·070	3·2
6·0	1·494	0·198	132·4	0·150	0·048	3·6
7·0	1·476	0·233	157·6	0·167	0·066	3·5
8·0	1·260	0·160	127·6	0·122	0·038	3·5
9·0	0·206	0·035	171·2	0·032	0·004	6·0

* Period of incubation in all cases was 35 days at 24°.

IV. *Nitrogen concentration of the medium.*

To determine the effect of varying the total available nitrogen upon growth and pigment production, quantities of the following two media without a source of nitrogen were made up: (*a*) glucose, 50 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; water to a final volume of 1 litre after addition of ammonium tartrate solution: (*b*) as (*a*) with 100 g. glucose in place of 50 g. 30 one litre conical flasks, each containing 340 ml. of medium (*a*) and 30 flasks containing 340 ml. of medium (*b*) were made up and sterilised. Each group was divided into 6 lots of 5 flasks each and to each were added 10 ml. of the appropriate sterile diammonium tartrate solution to give nitrogen concentrations equivalent to 1·0, 2·0, 4·0, 6·0, 8·0 and 10·0 g. of $NaNO_3$ per litre (1·0 g. $NaNO_3$ equivalent to 1·08 g. diammonium tartrate). The flasks were sown with equal amounts of a heavy spore suspension of *A. novus* (Ac 46) and incubated at 24° for 35 days. After this period the mycelia were filtered off, washed, dried *in vacuo* and weighed. The dried, powdered mycelium was thoroughly extracted and the pigments were separated as in the previous experiments. The results are given in Table VI.

Table VI. *Effect of variation in available diammonium tartrate upon the growth and pigment production of* Aspergillus novus (*Ac 46*)*.

Ammonium tartrate per litre of medium g.	Weight of mycelium per flask g.	Weight of crude pigment extract per flask g.	Crude extract per g. mycelium mg.	Crude flavo-glaucin per flask g.	Crude auro-glaucin per flask g.	Apparent residual glucose %
		1. Glucose content 5 %.				
1·08	0·807	0·097	119·7	0·070	0·027	3·27
2·16	1·287	0·255	197·9	0·185	0·070	2·21
4·32	1·587	0·303	190·9	0·224	0·079	1·29
6·48	2·043	0·538	263·5	0·427	0·112	0·368
8·64	2·185	0·813	371·9	0·672	0·140	0·368
10·80	2·310	0·997	431·7	0·764	0·233	0·368
		2. Glucose content 10 %.				
1·08	0·909	0·160	176·3	0·106	0·054	6·84
2·16	1·636	0·244	149·4	0·169	0·075	5·48
4·32	2·575	0·350	135·8	0·275	0·075	3·86
6·48	3·175	0·526	165·9	0·358	0·168	2·61
8·64	3·417	0·691	202·1	0·489	0·202	1·93
10·80	3·591	0·990	275·7	0·830	0·160	1·65

* Period of incubation in all cases was 35 days at 24°.

The most striking result obtained is that by increasing the available diammonium tartrate there occurs a very marked increase in growth, pigment production and glucose metabolised. With 10 % glucose, growth increases to a much greater extent than with 5 % glucose, the added increase in weight being essentially due to conidia formation since there is not a correspondingly great increase in pigment production. Since the weights of the total crude extracts are practically the same using 5 % or 10 % of glucose, it is obviously far more economical to use the lower concentration of sugar for the preparation of the pigments.

Using 5 % glucose and 10·8 g. per litre of diammonium tartrate the yield of pigment corresponds to 43·2 % of the total weight of mould substance and is equivalent to 5·64 % of the glucose metabolised. In spite of this remarkable increase in pigment production the ratio of flavoglaucin to auroglaucin still remains in the vicinity of 2·5–3·0. On this medium conidia formation is almost entirely absent, the growth being practically entirely perithecial.

A comparison of the growth obtained in these experiments with that obtained by Raistrick *et al.* [1931] on ordinary Czapek-Dox medium, containing 2·0 g. $NaNO_3$ as a source of nitrogen and using the identical organism in each case, shows that by the present modification in the medium, growth has been increased almost elevenfold, and three times the quantity of glucose has been metabolised.

V. *Concentration of glucose in the medium.*

Batches of flasks of the usual Czapek-tartrate medium containing respectively per batch 2·5, 5·0, 7·5, 10·0, 12·5, 15·0 and 20 % of glucose were made up and sterilised. Five flasks from each batch were sown with a heavy spore suspension of *A. novus* (Ac 46) and incubated at 24° for 35 days. The mycelia were then filtered off, washed, dried *in vacuo*, weighed, powdered and thoroughly extracted with ether and the pigments separated as previously described. From the results of the experiment given below in Table VII it appears that whilst growth

Table VII. *Effect of variation in glucose concentration upon the growth and pigment production of* Aspergillus novus (*Ac 46*)*.

% of glucose	Weight of mycelium per flask g.	Weight of crude pigment extract per flask g.	Crude extract per g. mycelium mg.	Crude flavoglaucin per flask g.	Crude auroglaucin per flask g.	Apparent residual glucose %
2·5	0·901	0·190	210·8	0·141	0·049	0·00
5·0	1·287	0·255	197·9	0·185	0·070	2·21
7·5	1·274	0·206	170·3	0·137	0·069	4·41
10·0	1·636	0·244	149·4	0·169	0·075	5·48
12·5	1·697	0·231	136·3	0·158	0·074	8·46
15·0	1·740	0·250	143·7	0·166	0·084	10·26
20·0	2·179	0·348	159·5	0·287	0·061	14·34

* Period of incubation in all cases was 35 days at 24°.

is increased when the glucose concentration is above 7·5 %, the total pigment production does not increase until 20 % glucose is available. It appears that with an increase in the available glucose there is a corresponding decrease in the percentage of pigment formed, due to the relative increase in conidia formation with the increase in glucose concentration.

VI. *Large scale preparation of flavoglaucin and auroglaucin.*

From the results of the previously described experiments on growth and pigment production by *A. novus* (Ac 46) the following modified medium was adopted for large scale production of flavoglaucin and auroglaucin: Solution I, glucose, 1750·0 g.; KH_2PO_4, 35·0 g.; KCl, 17·5 g.; $MgSO_4,7H_2O$, 17·5 g.; $FeSO_4,7H_2O$, 0·35 g.; distilled water, 32·5 litres. Solution II, diammonium tartrate, 378·0 g.; water, 2·5 litres. The solutions were made up separately, and 325 ml. of solution I were distributed in each of 100 one litre conical flasks and sterilised by steaming on 3 successive days at 100° for 1 hour. The diammonium tartrate solution was sterilised in the same way, and after sterilisation 25 ml. were added aseptically to each of the flasks. 385 flasks were made up in this manner and sown with a heavy spore suspension of *A. novus* (Ac 46). After incubation at 24° for from 30 to 35 days the flasks were removed, and the mycelium was filtered off, washed, dried and weighed. 767 g. of dried mycelium so obtained were powdered and thoroughly extracted with ethyl ether. The crude dry extract, 315 g., was then boiled with light petroleum (50–60°) and the insoluble crude auroglaucin filtered off (65 g.). The light petroleum solution was evaporated, and from this there separated 191 g. of crude flavoglaucin. The light petroleum mother-liquors on evaporation gave 59·0 g. of "crude fat."

Discussion.

Qualitative nature of pigment production. Earlier investigators have been of the opinion that pigment production by species in the *A. glaucus* series is quantitative as well as qualitative in the sense that any particular species in the series always produces the same pigments in the same final amounts. An analysis of Tables I and II might possibly lead one to conclude that there is a quantitative as well as a qualitative basis for the differentiation of the separate species in the series. It was found, however, in the present investigation that a comparison of the figures obtained in preliminary small scale experiments with those obtained in the final large scale experiments on the same organisms and under the same conditions showed no marked correlation. Some of the organisms gave in both cases comparable figures, whilst others showed an equally striking difference. It appears, therefore, that the present investigation indicates only a qualitative specificity of the pigments for the entire series and groups within it.

Final p_H *and pigment production.* In the experiment on the effect of p_H on the growth of and pigment production by *A. novus* (Ac 46), p. 1651, it was found that the optimum p_H was 5·4 and that growth and pigment production were very poor below p_H 5·4 and above p_H 7·0. It was also found that the final p_H in all cases where growth occurred tended to become lower than the initial p_H. In view of this finding the final p_H of most of the media used in the growth and pigment production experiments with *A. novus* (Ac 46) was determined, and it was found that most of the final p_H values fell within the limits 3·2–3·6, the exceptions being in those cases where growth and pigment production were markedly high. It seems, therefore, that one of the most important limiting factors for growth and pigment production is the p_H of the medium, and it appears that the proper buffering of the medium to maintain it at the initial p_H 5·4 is essential for increased growth and pigment production.

Earlier investigators were of the opinion that the walls of the stalks and aerial hyphae become encrusted with granules, yellow in the young colony when conditions are acid, and becoming red or ferruginous in age when the reaction

changes to alkaline. They assumed that the pigment behaved as an indicator to acid and alkali, yellow in the acid phase and red when alkaline. From the results of the present investigation it seems quite evident that both flavoglaucin and auroglaucin occur together and independently of the p_H. In the case of group III organisms, flavoglaucin and the red pigment occur together in spite of the acid nature of the medium. In growing *A. novus* (Ac 46) at various p_H values, both of the pigments could be isolated in every case where growth occurred, and the medium became acid after incubation rather than alkaline. Where the initial p_H was above 7·0 auroglaucin was present in lesser quantity than at the lower p_H values, which is contrary to what would be expected from the assumptions of earlier workers. It seems therefore that the colour of the colony is not an indicator of the reaction of the medium.

Site of auroglaucin and flavoglaucin formation. To show that the perithecia are the site of flavoglaucin and auroglaucin formation, attempts have been made to inhibit entirely the formation of perithecia, which appear to be the seat of pigment production because of their bright sulphur-yellow colour. In no case could their formation be completely suppressed, but when *A. novus* (Ac 46) was grown on the Czapek-tartrate medium at p_H 4 perithecia formation was greatly retarded. After 35 days' incubation at 24° the yield of crude pigment was 58·0 mg. per g. of mycelium as compared with the normal average obtained at p_H 5·4 of 197·9 mg. per g.

That the pigments originate in the perithecia seems to be indicated also by an analysis of pigment production during the early stages of growth. During the first five days of growth, when the foundation of hyphal threads is being laid down, and perithecia formation is scanty, the total crude pigment percentage of the mould substance is 9·9 %. During the following six days of growth perithecia formation is abundant and the growth becomes brilliant yellow. Correspondingly the total crude pigment increases to 33·8 % of the mould substance. During this period of abundant perithecia formation one-half the total increase in weight of the mould substance is accounted for by the increase in weight of pigment. Moreover, using the newly modified medium, the growth is practically purely perithecial and, correspondingly, the yield of pigment is 43·2 % of the weight of mould substance. That the pigment is not associated with the conidia seems quite definite, since during the period of abundant conidia formation, between the eleventh and thirteenth days, the total crude pigment extract per flask is practically constant, being 0·064 g. per g. of mould substance after 11 days and 0·066 g. after 13 days. It seems quite evident, therefore, that flavoglaucin and auroglaucin formation takes place in great part or entirely in the perithecia.

Rubroglaucin appears as red incrustations on the walls of the aerial hyphae.

The investigation has shown, therefore, that what has hitherto been considered a preliminary diagnostic or a confirmative character of species in the *A. glaucus* series is, actually, an absolute qualitative character of the series as a whole and of separate groups of species within the series.

Summary.

In a study of 25 species in the *Aspergillus glaucus* series, three hitherto undescribed crystalline pigments have been isolated from the dried mould substance: (*a*) flavoglaucin, $C_{19}H_{28}O_3$, in lemon yellow needles; (*b*) auroglaucin, $C_{19}H_{22}O_3$, in golden orange needles, and (*c*) rubroglaucin, $C_{16}H_{12}O_5$, in ruby-red short rods. These pigments are not produced by 19 other coloured species of *Aspergillus* examined and are believed to be specific for species in the *A. glaucus*

series. On the basis of these results the entire *A. glaucus* series has been divided into three distinct pigment groups. Flavoglaucin has been isolated from all 25 species in the *A. glaucus* series and is, therefore, a series-specific pigment. Group I contains thirteen species which produce flavoglaucin and auroglaucin but no other pigments. Group II contains two species which produce flavoglaucin, auroglaucin and an unidentified red pigment. Group III contains 10 species which do not produce auroglaucin but produce, in addition to flavoglaucin, a red pigment. In four species in this group this pigment has been shown to be rubroglaucin. In the remaining six species the red pigment has not been identified. The optimum cultural conditions for the production in quantity of flavoglaucin and auroglaucin by *Aspergillus novus* Wehmer have been determined.

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CCXIX. MILK SECRETION IN RELATION TO BLOOD COMPOSITION. THE UREA CONTENTS OF BLOOD AND OF MILK-SERUM.

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THE following experiments deal with the urea contents of milk, arterial blood and mammary venous blood and with the rate of milk secretion. Though only intended to be preliminary to a series of investigations concerning the rate of elimination of various constituents in milk, they have been recorded here, together with the lines for future work which they indicate, since the author is unable to continue them at present.

The work has been carried out by the use of methods which have been very similar to those that are generally employed in determination of the blood-urea clearance by the kidney [see Peters and Van Slyke, 1932]. The udder of each experimental cow was first thoroughly emptied at the usual hour of morning milking. The cow was then thoroughly milked out on two occasions, the intervals between these three milkings (1 to 2 hours each) being measured accurately. The yield of milk was weighed on a spring balance recording to 1/100 lb. A blood sample from the abdominal subcutaneous (mammary) vein was taken a few minutes after the middle milking.

The specific gravity and fat content of the milk samples (by hydrometer and the Gerber methods, see Ling [1930]) and the urea contents of the whole blood and whole milk samples (by the method of Peskett [1934]) were determined. From the data, the urea content of the fat-free serum of the milk samples was calculated assuming no urea to be contained in the fat; also the rate of secretion of fat-free serum for each of the two test periods. The results are shown in Table I.

Table I. *Rate of secretion and urea content of milk-serum and blood.*

	Duration of test periods (mins.)		Rate of secretion (ml. milk-serum/min.)		Urea content of milk-serum (mg./100 ml.)		Urea content of whole blood (mg./100 ml.)
Name of cow	Period 1	Period 2	Period 1	Period 2	Period 1	Period 2	
Gertie 3	72	83	4·4	4·6	28	29	28
Peach 3	69	83	2·5	13·7	28	34	33
Rosey G	78	94	4·2	9·9	35	40	37
Winsome 2	79	90	3·8	7·5	33	33	29
Pearl	79	91	10·0	5·5	44	45	44
Lottie 8	85	100	10·3	8·7	36	36	38
Fanny 2	95	118	18·2	6·4	38	37	41

As might be expected, urea being freely diffusible, the urea contents of blood and fat-free milk-serum are identical within the limits of experimental error, and this relation is unaffected by the rate of milk secretion. It may be noted that the largest difference between blood and milk-serum urea contents occurred

in Period 1 of the experiment on Peach 3, when the rate of secretion was very small; the smallest, indeed, of the series.

No significant differences have been observed between the urea contents of blood samples from the mammary vein and from the internal iliac artery. These have been examined in a different group of cows (Table II).

Table II. *Urea content of bovine arterial and mammary venous blood (mg./100 ml.).*

Name of cow	Peggy	Flora 11	Flora 18	Cora 1	"Phosphor"	"Phosphor"
Arterial	19	18	21	45	27	29
Mammary venous	17	19	22	43	25	31

The experiments described in this note show that urea is not concentrated by the mammary gland as it is by the kidney. Further, they suggest that the blood-urea of the cow can be determined as well on the fat-free serum of milk as on the blood itself. If, as seems probable, the same conclusions apply to all mammals, then a simple alternative method of determining blood-urea is available during lactation, obviating the need for withdrawal of blood samples.

As continuation of this work I had planned to investigate the elimination rate of other milk constituents and, at the same time, to determine their concentration in mammary venous and iliac arterial bloods, thus combining the two types of experiment recorded above. By comparing the absorption of calcium and potassium, for example, from the blood flowing through the mammary gland with the amount of their secretion in the milk-serum, I hoped to calculate the volume of blood which must pass through the udder to produce a given volume of milk. This problem has so far received attention only, as in the work of Lintzel [1934], in experiments dealing with the alleged milk precursors absorbed from the blood, which obviously undergo complex metabolic change in the gland and may appear in the milk in a different phase, *e.g.* the fat. In addition, I anticipated obtaining information of the rate of blood flow through the udder and of its relation to the rate of milk secretion, a major problem of lactation on which no information is at present available.

SUMMARY.

In the cow, whole blood and the fat-free serum of milk have the same urea contents irrespective of the rate of milk secretion. Mammary venous blood is not significantly different from arterial blood in this respect.

During lactation milk may be used as an alternative to blood for determination of blood-urea.

A method is suggested for estimating the volume of blood which participates in the formation of a given volume of milk and for determining the approximate rate of blood flow through the mammary gland.

I am indebted to Capt. W. L. Little and Dr W. R. Graham for collection of the arterial blood samples.

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CCXX. A STUDY OF THE IMMUNOLOGICAL ACTION OF TYROSINASE FROM MEALWORMS.

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The problem of the production of melanin in mammals has of recent years been regarded mainly, in consequence of the work of Bruno Bloch, as the problem of determining the conditions of conversion of dihydroxyphenylalanine into melanin by a dopa-oxidase. The actual presence of free dihydroxyphenylalanine in human skin has, however, of late been considered doubtful; and on the other hand Schmalfuss *et al.* [1933] have established, by isolation and chemical identification, the normal occurrence of *l*-tyrosine in a free state in human skin, even before any *post mortem* autolysis. The significance of tyrosine and of the tyrosinase requisite to convert the amino-acid into a black pigment appears therefore to be enhanced as a possible explanation of the formation of melanin. The presence of tyrosinase in mammals is, as is well known, not easy to demonstrate. Even in lower animals, as for example in mealworms, the presence of some inhibitory factor along with the tyrosinase is evident. Mealworms, when freshly dissected, are not to any degree darkened, and injury to the tissues is necessary before the powerful melanogenic activity of the cells when ground up is revealed. Knowledge of any factor inhibitory to tyrosinase would consequently appear to be of some importance in the problem of the production of melanin. In order to study such factors, we have attempted to produce by immunisation specific antibodies to mealworm tyrosinase.

The attempt to produce an antityrosinase is not new; Gessard [1901], many years ago, reported success using tyrosinases of plant origin. Von Fürth [1912], on the other hand, reported negative results. We describe here our own observations.

Experimental.

Tyrosinase was prepared by grinding up 100 g. of mealworms in a litre of phosphate buffer mixture, p_H 8, and centrifuging the crude extract. The supernatant liquid was discarded so as to avoid the presence of somewhat toxic dissolved substances. The deposit was again shaken with the buffer mixture. Tyrosinase from the deposit was found to go into solution and give active preparations. This solution was then centrifuged and kept with thymol until required. Immediately before injection the solution was centrifuged and filtered through filter-paper to remove fat and suspended particles. After adjustment to make the solution approximately isotonic it was injected a few ml. at a time, four or five times, at intervals of four days. The injections were made slowly, and no appreciable disturbance was noted among the animals. Ten days after the last injection, blood was drawn and allowed to clot for the collection of serum.

It was noticed first of all, in the preliminary controls, that any fresh serum from an unprepared rabbit has in itself some inhibitory effect on the production of melanin when added to an active tyrosinase from mealworms. But unquestionably the serum from a prepared animal is more inhibitory for an active tyrosinase than any unprepared serum.

To the same quantity of tyrosinase (0·5 ml.) in a number of test-tubes increasing quantities of the sera under examination from 0·005 ml. to 0·5 ml. were added. The volume in all the tubes was equalised by addition of phosphate buffer, p_H 8, and the mixtures were left to stand for ten to twelve hours at room temperature with some thymol to prevent infection. Ten ml. of a solution of tyrosine in phosphate buffer, p_H 8, were then added to every tube and mixed. Under these conditions the production of melanin was very slow, spread over many days even in the controls, an effect which was found convenient. The differences were easily noted, and the slow uniform darkening was readily estimated quantitatively with the aid of a colorimeter, the pigment remaining in suspension in a completely colloidal state. The intermediate red colour was found not to interfere at all in these experiments. The intensity of the production of melanin could be plotted in arbitrary colorimetric units by comparison with the colour of the tube in which enzyme and tyrosine are present in phosphate buffer in the same concentrations but with no serum whatever. This colour was called arbitrarily 100 units. The results of such experiments are given in Table I. It was of interest to compare albino with coloured animals in this connection. Any difference between grey and albino rabbits appeared to be fortuitous. Similarly in a number of other experiments albinos gave no exceptional result. The purity of an albino strain is however difficult to ascertain with certainty.

Table I. *Showing the inhibition produced by normal serum and a serum of an animal (albino) injected with active tyrosinase.*

Both sera are inhibitory for an active tyrosinase but the normal in a lesser degree for equal concentrations.

Serum used	Amount of serum added to 0·5 ml. of tyrosinase ml.	Melanin produced in arbitrary units	Remarks
Normal	0	100	Tyrosinase active
	0·0054	97	? Inhibition
	0·012	101	No change
	0·0236	91	Inhibition
	0·059	79	Marked inhibition
Of an animal injected with active tyrosinase	0	100	Tyrosinase active
	0·0018	118	Slight activation
	0·0054	86	Inhibition
	0·0236	64	Marked inhibition
	0·059	77	Inhibition less marked when the quantity of added serum becomes excessive

The above results were those obtained when an active tyrosinase was injected into rabbits, and the sera were tested on an active tyrosinase.

It was noticed that the preparations of tyrosinase in phosphate buffer, p_H 8, prepared as described above were not very stable. Their activity decreased more quickly than that of the usual extracts in dilute ammonia, although chloroform or thymol was used as antiseptic. When, after an interval of six days, the experiments described in Table I were repeated using the same tyrosinase and sera, the tyrosinase in the controls was found to be very much weaker. The

results on addition of sera were however surprisingly different. The sera initially used which had proved to be inhibitory for strongly active tyrosinase preparations, at the same concentrations and under the same conditions, were now powerful activators of the feebly active tyrosinase preparations. There was, in fact, a complete inversion of the previous phenomenon. An example of this effect is recorded quantitatively in Table II.

Table II. *Showing the activation of a tyrosinase which has lost very much of its original activity when sera are added.*

The serum of an albino prepared by injections of active tyrosinase exhibits a greater power of reactivation for equal doses than a normal serum.

Serum used	Amount of serum added to 0·5 ml. of tyrosinase ml.	Melanin produced in arbitrary units	Remarks
Normal (albino)	0	100	Tyrosinase of a very poor activity
	0·0018	110	Tyrosinase somewhat activated
	0·0054	125	Activation
	0·012	127	Activation
	0·0236	170	Activation
	0·059	201	Marked activation
Normal (dark rabbit)	0	100	Tyrosinase of a very poor activity
	0·0018	100	No activation
	0·0054	114	Slight activation
	0·012	139	Activation
	0·0236	166	Marked activation
	0·059	140	Activation less pronounced when the quantity of serum is too large
Serum of an albino injected with active tyrosinase	0	100	Very poorly active tyrosinase
	0·0018	158	Considerable reactivation even with minute traces of serum
	0·0054	198	Very marked activation
	0·012	228	Very marked activation
	0·0236	252	Very marked activation
	0·059	229	Activation somewhat decreased when the amount of added serum is too large

The fact that the inversion was due simply to the feebleness of the tyrosinase was readily established by testing the same sera again with an active tyrosinase. Inhibition of the production of melanin was observed as previously. Repetition with a feebly active tyrosinase gave on the other hand an activation of the enzyme.

A preparation of tyrosinase (in the usual phosphate buffer, p_H 8), the activity of which had become so feeble that it might be considered practically inactive, was then injected into other rabbits. This preparation of tyrosinase had been kept with the antiseptics mentioned and in the concentrations described for the first experiment. It gave hardly a trace of melanin after two weeks. The serum prepared by use of a feebly active tyrosinase, however, appeared to be a powerful activating agent for any feeble tyrosinase; melanin was produced after four or five days and continued to accumulate. On the other hand the serum had no appreciable inhibitory effect on an active tyrosinase, such as is exercised by a normal serum, as already stated.

The reactivation of a practically inactive tyrosinase by a serum of a rabbit injected with a practically inactive tyrosinase is illustrated in Table III.

Table III. *Showing the reactivation of an inactive tyrosinase by a serum of an animal prepared by injections of the inactive enzyme.*

The inactivity of the enzyme was due to ageing.

Serum added	Amount of serum added to 0·5 ml. of tyrosinase ml.	Melanin produced (arbitrary units)	Remarks
Normal	Various quantities of normal serum could not reactivate the solution of tyrosinase which was practically inactive		
Serum of an animal prepared by injections of inactive tyrosinase	0·025	102	No reactivation
	0·05	107	Trace of activation
	0·1	113	Trace of activation
	0·2	158	Marked reactivation
	0·4	180	Marked reactivation

A final experiment was made with two rabbits which were injected with the following preparations in the order given: (1) a fairly active tyrosinase, (2) a feebly active tyrosinase, (3) and (4) a tyrosinase with activity between (1) and (2). The effects of the sera thus prepared varied with the amount added to the tyrosinase-tyrosine mixture. On testing with a feebly active enzyme the latter was activated by small or intermediate amounts of the serum but inhibited by larger amounts. This inhibition is probably due solely to the large amount of serum added and occurs with any serum, normal or prepared.

Certain additional observations were made. Heating the prepared sera to 55° for about a quarter of an hour was found to change their properties. The activating effect on a feebly active tyrosinase was diminished but the inhibitory effect of high concentrations of the serum was abolished (see Table IV). This inhibitory effect likewise disappeared when the serum was kept with thymol for some months, the reactivating power on feeble tyrosinase preparations, however, remaining unaffected.

Table IV. *Differences in the reactivations of a poorly active tyrosinase by a serum of an animal prepared by injections of active tyrosinase when the serum has been heated to 55° for a quarter of an hour.*

Amount of serum to 0·5 ml. of tyrosinase ml.	Melanin produced	
	A. Serum unheated	B. Serum heated to 55°
0·0018	158	102
0·054	198	113
0·012	228	129
0·0236	252	151
0·059	229	203

Discussion.

It appears from these experiments that the response of a rabbit to injections of tyrosinase from mealworms is different when the injected enzyme is active and when it is feeble or even practically inactive. When the injected enzyme is very feeble, or inactive, a feebly active tyrosinase is reactivated by the serum, and no appreciable effect is observed when the serum is tried on an active tyrosinase.

When the injected enzyme is active, the effect of the serum is different according as the tyrosinase with which it is tested *in vitro* is active or feeble.

Such serum is inhibitory towards an active tyrosinase and reactivates a feeble one.

In attempting to elucidate the complex phenomena observed, it would appear to be helpful to postulate two factors in the usual solutions of tyrosinase, the enzyme itself and an antagonist to its activity.

Graubard [1933] came across inhibitors to tyrosinase in *Drosophila melanogaster*. He found that extracts of the larvae and pupae of this insect exhibited inhibitory phenomena, but by direct observation did not find the same phenomena with mealworms. We consider that the existence of an inhibitor in mealworm tyrosinase is suggested by our experiments in view of the following considerations.

Assuming the presence of an antagonistic factor in the usual tyrosinase preparation, both the enzyme itself and the antagonist may be able to develop specific antibodies when injected into the circulation of a rabbit. Suppose now the prepared serum be allowed to react with two sorts of tyrosinase, one active and the other feeble. A very active preparation of tyrosinase may be regarded as a mixture in which the amount of enzyme is relatively greater than that of the antagonistic factor. If it be assumed that in contact with the prepared serum, on account of the relatively greater masses of the reactants, the neutralisation of the active part of the enzyme preparation will be more readily achieved than any other combination then a reduction in activity of the enzyme by this neutralisation would be the expected result. This would explain the decrease in melanogenic power of the active enzyme which is illustrated in Table I. It may be noted that when the concentration of serum for the same quantity of tyrosinase increases (more than 0·0236 ml. in the particular experiment quoted) the amount of the second supposed antibody (specific to the inhibitory factor in the active tyrosinase preparation) would seem to be great enough to react with the antagonistic factor and to neutralise it partly, with a resulting decrease in the inhibitory effect on melanin formation observed with smaller concentrations of serum.

A tyrosinase which has become feebly active may be regarded as a solution in which the concentration of the enzyme is now relatively less than that of the antagonistic factor. The neutralisation of the latter by the serum will therefore, on account of the greater masses of the reactants, be more readily achieved than any other reaction. The result will be a development of melanogenic power following from the neutralisation of the antagonistic substance. This would explain the phenomena recorded in Table II.

When a solution of tyrosinase ages and becomes inactive, it would seem that all the enzyme has disappeared by autolysis or has otherwise undergone destruction. The antagonistic factor may nevertheless remain, and when injected, the solution would now be able to produce antibodies to the antagonistic factor only. (Numerous observations have established that such antibodies are the more stable with the lapse of time and are still active when the antibodies specific to the enzyme have become inactive.) This would explain the fact that the serum of an animal injected with inactive tyrosinase is a powerful reactivator of the feeble enzyme preparation, in contrast to the normal inhibitory effect of an unprepared serum on an active tyrosinase.

It is noteworthy that the fresh serum of an unprepared rabbit is a definite though weak inhibitor for an active tyrosinase, and a definite though weak reactivator for a feeble tyrosinase. The preparation of rabbits by injections of tyrosinase enhances greatly these latent properties. But whether this is due to the production of some substance already present in normal serum or to formation of a new inhibitor cannot be settled by these experiments.

Finally, apart from the particular interest attached to tyrosinase itself, it might be of use to test a number of enzymes for the production of effective activators in the way described for tyrosinase. The injection of the inhibitory constituent of an enzyme preparation might be a general method for the activation of an enzyme by means of the antibodies produced.

SUMMARY.

1. The injection of an active tyrosinase preparation into rabbits modifies the properties of the animal's serum.

2. The prepared serum, when the injected tyrosinase is active, has an inhibitory effect on a tyrosinase preparation if this is strongly active, but it reactivates a tyrosinase which is of poor or even very feeble activity.

3. The prepared serum, when the injected tyrosinase is very feeble or practically inactive, has no effect on an active tyrosinase. The same serum is able to reactivate a feeble or practically inactive tyrosinase.

4. A possible interpretation of the facts observed is that two antibodies are generated, specific to the enzyme and to a presumed antagonistic factor in the enzyme preparations; this suggestion is discussed.

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CCXXI. THE INFLUENCE OF THE SOLVENT ON THE BIOLOGICAL EFFECT OF CAROTENE AND VITAMIN A.

By KATHLEEN CULHANE LATHBURY and GEORGIANA NORA GREENWOOD.

From the Physiological Laboratories of The British Drug Houses, Ltd.

(*Received July 27th, 1934.*)

Since it was first shown by Euler *et al.* [1928] that carotene could replace vitamin A in causing the resumption of growth of rats depleted of their reserves of this vitamin, experiments have been described in which the amount of carotene stated as being capable of restoring growth has shown considerable variations. These differences were partly but not entirely dispelled when Drummond *et al.* [1930] found that the instability of carotene in ethyl oleate, the solvent employed by them in their original biological tests [Dulière *et al.*, 1929], accounted for its apparent ineffectiveness. Moore [1933] published some experiments in which he tried to define the "minimum doses" of carotene and of vitamin A; but his figures only represented "minimum doses" for the particular diet and technique employed by him. Since the experiments of Drummond *et al.*, it has been repeatedly confirmed that certain oils are quite unsuitable for the biological test. Smedley-MacLean and Hume [1930] and Drummond *et al.* [1930] concluded that the observed discrepancies were due to the rapid oxidation which carotene undergoes in certain solvents. Notwithstanding this, we have found that when oils are used in which carotene is reasonably stable, there is still a wide divergence in the quantitative results obtained both with carotene and with vitamin A, so that the rate of deterioration of the vitamin A or carotene does not afford a sufficient explanation of the results, and the suitability of an oil from the physiological point of view is a separate problem. Failure to realise this biological influence of the solvent explains some of the difficulties which workers have experienced in the estimation of vitamin A in terms of the International Standard carotene, and in November 1932 we communicated our results to the Vitamin A Sub-Committee of the Accessory Food Factors Committee. Dr Coward, working on behalf of the Committee, has confirmed many of them, and at their request we made an experiment comparing three selected coco-nut oils, the result of which is described in this paper.

In a paper published by one of us in 1933 [Culhane, 1933] attention was drawn to the fact that rats reared on the selected basal diet gave no response to a dose of 0·002 mg. of carotene dissolved in arachis oil, which was considered a sufficient dose by Moore. We also found it necessary to give as much as 10 times the dose of cod-liver oil recommended by Coward for use with her diet. Nelson and Jones [1928], using a diet containing very highly purified caseinogen, had published experiments which showed effective doses of about the same magnitude as ours. We concluded that the wide diversity in the quantity of vitamin A-containing substances necessary to restore growth was due to the differences in the

basal diet employed. A number of experiments in which the constituents of the basal diet were systematically varied, afforded valuable information regarding the incidence of xerophthalmia, but they failed to provide an explanation of the need for such very different quantities of vitamin A to be associated with the various diets. In continuation of these experiments we tried the effect of different oils as solvents and found that the result of varying these was much greater than that of changing any other constituent.

An experiment showed coco-nut oil to be greatly superior to arachis oil as a solvent for carotene. We followed this observation by quantitative comparisons of various oils used to dissolve both carotene and vitamin A concentrates, and we found that not only do oils differ, but different samples of the same oil may vary greatly in their suitability quite independently of the rate of decomposition of the substance dissolved. Certain samples of arachis oil we have found to give excellent results, whereas certain samples of coco-nut oil proved quite unsuitable.

The diet used throughout has been the same as the standard diet used in the paper referred to above (A 5 diet). The litters used in each comparison have been as large as possible, and only litter-mates of the same sex have been compared. The experiments have been closed after the rats have received oils for ten weeks, and average growth curves have then been constructed for comparable groups of rats. When an animal has died in the course of a test, we have omitted it and its litter-mates from the final result. We consider that this is the only justifiable procedure, since a dead animal cannot take part in a growth test. Upon occasions, however, when a number of rats have died, in addition to the comparison of the average growth curves of the survivors, we have made a comparison of the average periods of survival on the different doses.

Both in the case of carotene and vitamin A it is our practice to make a sufficient amount of the various dilutions for feeding to last for about five weeks. They are poured into small amber bottles containing about $2\frac{1}{2}$ g. and corked, care being taken that the bubble of air at the top of the bottle is as small as possible. The bottles are immediately placed in the cold room, and the one in use for feeding each day is returned to the cold as soon as possible. One bottle usually lasts two or three days only, but if there are only a few animals on test it lasts longer; in any case it is discarded and a fresh sample taken after one week.

Experimental.

A. *Comparison of arachis oil and coco-nut oil.*

(1) *As solvents for carotene.* The carotene employed consisted of a mixture of isomerides estimated to contain about 85 % of β-carotene. It melted at 177–179°. 85 rats were used in the comparison and the doses given were 1γ and 2γ in 0·02 ml. of each solvent.

In Fig. 1 (1) and (2), the average growth curves for groups of 7 male and 4 female litter-mates show clearly that with either solvent it is easy to distinguish between doses bearing a ratio of 1 : 2. 2γ in arachis oil has given the best growth, 1γ in arachis oil and 2γ in coco-nut oil are about equally effective and 1γ in coco-nut oil has given the least growth. The average curves of groups of 9 male litter-mates (Fig. 1 (3)) confirm the equivalence of 1γ in arachis oil and 2γ in coco-nut oil.

Hence of these two, the arachis oil is about twice as effective as the coco-nut oil.

(2) *As solvents for vitamin A.* In another experiment using different batches of oils, coco-nut oil and arachis oil were compared as diluents for a vitamin A

concentrate—Carr-Price figure of 49,000[1] and $E^{1\%}_{1\,cm.} = 1100$. For feeding to the rats it was diluted with the oils under test to give values of 4 and 8 blue. 75 rats were used for the comparison. Fig. 2 gives the average growth curves of comparable groups of rats. Both males and females on the smaller and larger doses have given better results with the coco-nut samples. Hence, of these two, the coco-nut oil is superior to the arachis oil.

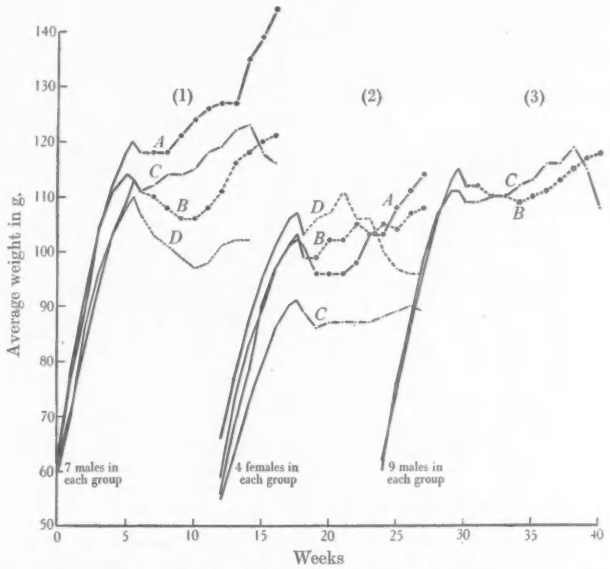

Fig. 1. Average growth curves for groups of rats (litter-mates) "run out" on A 5 diet. When growth ceased they were given daily doses of 0·02 ml. of solutions of carotene in arachis or coco-nut oils.

A. •—• 2γ daily in arachis oil *C.* ·—· 2γ daily in coco-nut oil
B. •---• 1γ „ „ *D.* ····· 1γ „ „

At the same time a few rats were given a dose of 4 U of standard dissolved in the coco-nut oil. Their average growth curves are shown in Fig. 3. Apart from the fact that they are so few in number, the comparison gives a fair assay of the vitamin A content of the coco-nut oil sample, the effect of the solvent having been eliminated. From Fig. 3 one can gain an idea of the very different results that would be obtained by assaying the vitamin A content of these concentrates in the arachis oil or in the coco-nut oil. The latter would give a result of 1,225,000 U/g., the former not more than 612,500 U/g. Further, had the standard been given in the arachis oil, the coco-nut oil solution would have appeared to contain 2,450,000 U/g.

(3) *Arachis and coco-nut oils compared with linseed oil and ethyl oleate.* Two other batches of these two oils were compared with each other and with ethyl oleate and linseed oil, using 102 rats in a test of the potency of a vitamin A

[1] All "blue' figures quoted in this paper were determined by the method described in British Pharmacopoeia 1932, p. 596, making appropriate allowances for dilution.

preparation giving a Carr-Price value of 23,000. The concentrate was diluted in the ratio 1 part in 4600 for test and the usual dose, 0·02 ml. of the solution, was fed daily to each animal.

The rats receiving their doses in ethyl oleate declined steadily, and all were dead long before the ten weeks of the experiment had passed. The growth was not very satisfactory on any of the oils and there were a number of deaths.

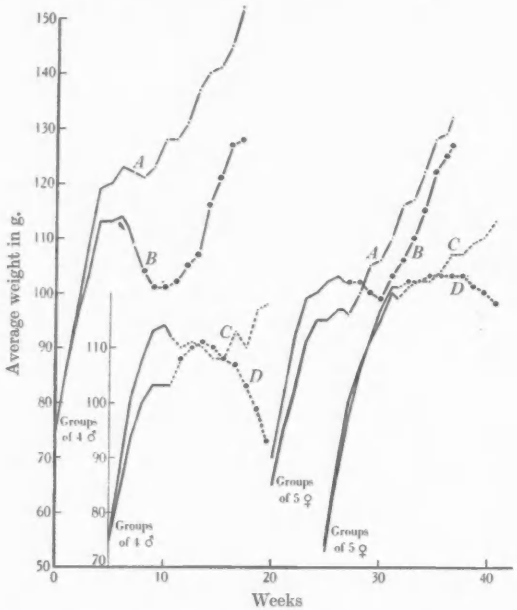

Fig. 2. Average growth curves for groups of rats (litter-mates).

A.	·——·	Vitamin A concentrate diluted to 8 blue with coco-nut oil; 0·02 ml. daily.								
B.	•——•	,,	A	,,	,,	8	,,	arachis oil	,,	,,
C.	- - - - -	,,	A	,,	,,	4	,,	coco-nut oil	,,	,,
D.	•- - -•	,,	A	,,	,,	4	,,	arachis oil	,,	,,

Fig. 4 shows some of the comparisons between groups of litter-mates and it follows from them that the linseed oil has given the best growth, the arachis oil being slightly superior to the coco-nut oil.

In yet another comparison of two further specimens of coco-nut and arachis oils, in which vitamin A was dissolved at two different dose levels, we found the oils about equally effective whether the comparison was made by means of the growth rate of the survivors or by comparing the rate of mortality of those which died.

B. *Comparison of three samples of coco-nut oil.*

The comparison of three samples of coco-nut oil was carried out at the request of the vitamin A Sub-Committee of the Accessory Food Factors Committee. Two of the oils were from Cochin (referred to as Cochin 1 and 2), and the third from Ceylon.

152 rats (19 litters) were used in the comparison of these oils. The standard was dissolved in each at a level of 100 units per g. so that the rats received 2 units per day. All doses were duplicated by making up additional solutions containing 0·01 % of quinol. The rats were distributed as evenly as possible between the six doses, one or two rats from every litter being left as negative controls. In Fig. 5 are shown the comparisons of the solutions containing no quinol: they show that the Cochin 1 oil is definitely less effective than the other two. Those of

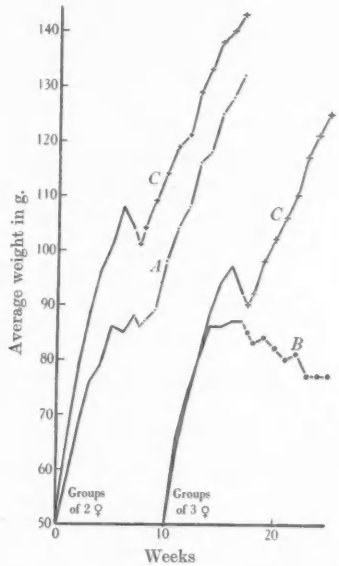

Fig. 3. Average growth curves for groups of rats (litter-mates).

A. ·—· Vitamin A concentrate diluted to 8 blue with coco-nut oil; 0·02 ml. daily.
B. •—• ,, A ,, ,, 8 ,, arachis oil ,, ,,
C. ×—× 4 units of standard carotene in 0·02 ml. of coco-nut oil daily.

the solutions with quinol are similar but are omitted for the sake of space. The order of the two superior oils is somewhat indefinite, but there is no doubt that the Cochin 1 oil is inferior, and the rate of mortality confirms the fact that it is less satisfactory than the other two oils. Thus, on this oil, twenty died out of twenty-five, on the Cochin II oil, one died out of thirty-one, and on the oil from Ceylon, five died out of twenty-seven.

In comparing the solution containing quinol with those that did not, we had groups of twelve litter-mates on each of the six samples. With every pair the result was the same on the males and females, there being a slight difference in favour of the oils without quinol.

The chemical properties in which the Cochin 1 oil differed from the other two are shown in Table I, but none of these differences was sufficient to account for the different actions of the oils.

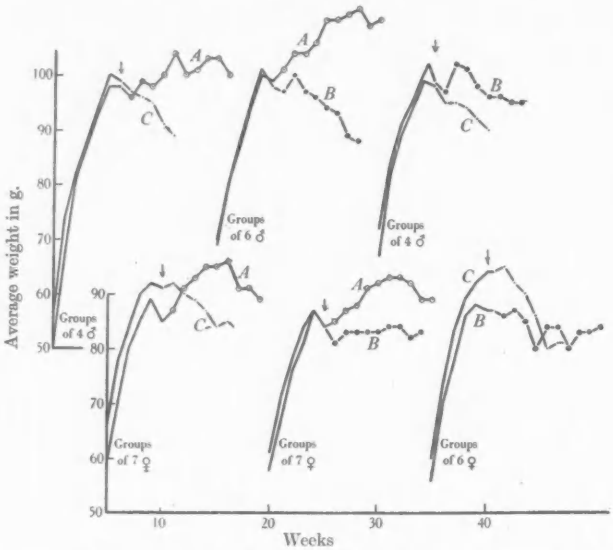

Fig. 4. Average growth curves for groups of rats (litter-mates). After "running out" on A 5 diet they were given 0·02 ml. daily of vitamin A concentrate diluted 1 part in 4600 (*i.e.* to 5 blue) with different oils:

A. ⊙—⊙ Linseed oil *B.* •—• Arachis oil *C.* ·—· Coco-nut oil.

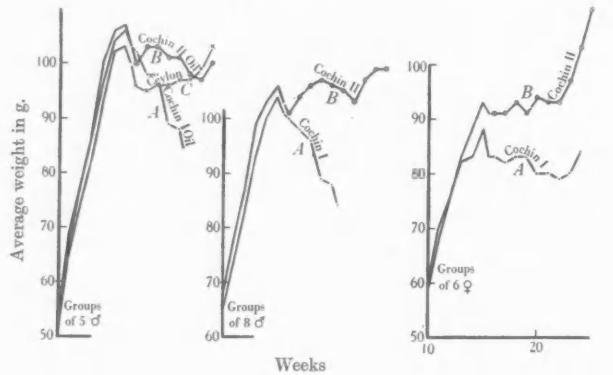

Fig. 5. Average growth curves for groups of rats (litter-mates). After "running out" on A 5 diet they were given 2 units of international standard carotene in 0·02 ml. of three different coco-nut oils:

A. ·—· Cochin I oil. *B.* ⊗—⊗ Cochin II oil. *C.* ×—× Oil from Ceylon.

Table I*.

	Coco-nut oils		
Colour	Cochin I White	Cochin II Slightly coloured	Ceylon Slightly coloured
Acid value	3·0	3·5	3·4
Iodine value	9·1	8·3	8·0
Peroxide test, ml. $N/200$ thiosulphate	0·05	0	0
After 190 hours' exposure to 60 Watt lamp	0·35	0·35	0·40
After 840 hours' exposure to 60 Watt lamp	0·70	1·60	1·60
Repeated 4 months later	0·10	0·05	0·05

* For these figures and for other chemical estimations quoted in this paper we are grateful to the Analytical Department of The British Drug Houses, Ltd.

Discussion.

Considering only the results with the different coco-nut and arachis oils, which are the varieties of oils most commonly used in this country in the testing of vitamin A, we have obtained the following results:

A. *As solvents for carotene.*

(1) Original experiment (not described here).
The coco-nut oil was superior to the arachis oil.
(2) Quantitative test (described above).
Carotene dissolved in arachis oil was twice as potent as in solution in coco-nut oil, different batches of the oils being used.

B. *As diluents for vitamin A concentrates.*

(1) Vitamin A dissolved in coco-nut oil was more effective than when dissolved in arachis oil.
(2) A vitamin A concentrate dissolved in another arachis oil was superior to its solution in coco-nut oil (linseed oil was better than either, ethyl oleate was greatly inferior).
(3) A vitamin A concentrate was equally active when tested in arachis oil or in a coco-nut oil. (Not described.)

The most obvious suggestion to explain these apparently contradictory results is that carotene and vitamin A deteriorate more rapidly in some oils than in others, but colour tests, where these have been carried out, show that this is not the true explanation. Unfortunately, the earlier experiments were not checked by colour tests, but we have sufficient data to show that there is some other factor involved.

Considering the pair of carotene solutions in coco-nut oil and arachis oil, there appeared to be no difference in their colours, either when they were first made up, or after the test had been proceeding for some weeks. Further, there was no sudden spurt of growth when they were made up freshly, which one would have expected of the coco-nut oil solution if its comparative inactivity had been due to more rapid deterioration of carotene. Colour tests on some of the same carotene dissolved in coco-nut oil carried out six months previously had shown only a very slight loss in four months: 4·8–4·5 in the solution containing 100γ per g., and 2·5–2·2 in the solution containing 50γ per g. In all probability these differences are not significant in view of the error inherent in the tintometer reading.

When carotene was again tested in the three different coco-nut oils, we carried out colour tests on the actual solutions used. These are shown in Table II, from which it can be seen that none of the samples had suffered any change whatsoever in six weeks. These figures show that our method of keeping carotene solutions is satisfactory, that quinol is unnecessary, and that we must seek for some other explanation of the differences between the oils than that of the deterioration of the carotene.

Table II. *Yellow colours read in the Lovibond tintometer.*

0·01 mg. carotene in 0·1 g. of oil diluted to 2 ml. with chloroform.

Oil	At 2 weeks	At 8 weeks
Cochin I	4·6	4·8
Cochin I + 0·01 % quinol	4·8	5·3
Cochin II	5·2	5·8
Cochin II + 0·01 % quinol	5·6	5·9
Ceylon	4·9	5·1
Ceylon + 0·01 % quinol	4·4	4·8

In the comparison of coco-nut oil, arachis oil, linseed oil and ethyl oleate as solvents for vitamin A, colour tests after the conclusion of the experiment showed that all had lost significantly, the ethyl oleate sample giving a Carr-Price value of 1·2 blue representing a loss of three-quarters of its vitamin content. Although this is a very substantial loss, it is our experience that rats receiving a dosage at so low a level as 1·2 blue would have given some response to the dose. However, it is not possible to draw conclusions from absolute values but only from comparisons. We found that the linseed oil and the arachis oil solutions were equal by the colour test, both having lost about one-third of their value, but the result of the growth test was far removed from equality. Referring again to Fig. 4 there is no doubt of the superiority of the solution in linseed oil. The coco-nut oil sample had lost about half its value by colour test, which shows a sufficient difference in vitamin A content to account for its inferiority.

Baumann and Steenbock [1933] have shown that quinol retards the decoloration of carotene in coco-nut oil, and the stabilising power of quinol for vitamin A solutions is well known. Thus, quite apart from our observation that the carotene solutions did not change over a period of six weeks, if the deterioration of the dissolved substance had been the cause of the differences that we have found, then the solutions of carotene in the inferior oil to which quinol was added should have given a growth result better than that obtained without addition of quinol and equal to that obtained with the other two oils. In actual fact this sample containing quinol gave the worst result of all.

The fact that sometimes the sample in arachis oil is the better and sometimes the one in coco-nut oil, whether carotene or vitamin A concentrate be used for the vitamin, precludes the possibility that carotene, before it can replace vitamin A, needs supplementing with some substance generally found in one of the varieties of oil. That an oil which allows the best growth may contain some factor necessary to supplement these highly purified preparations of vitamin A and its probable precursor is not unlikely, but if that is the case, the experiments show that it is not necessarily present in oils of any one kind. If this is the explanation, the substance is present in the sample of linseed oil tested, but absent from ethyl oleate; it is more abundant in one arachis oil than in the coco-nut oil with which it was compared, whilst with another pair the reverse is true. Further, of the three coco-nut oils compared directly, one contained much less than the other two.

We are continuing this work with a view to finding some physical or chemical means of selecting an oil that will be satisfactory in the biological test, and also to discovering the cause of the difference to the animal of oils that appear to be similar.

SUMMARY.

1. Different batches of arachis oil and of coco-nut oil vary in their suitability as solvents for the biological testing of vitamin A or carotene.
2. The suitability of a particular oil can only be determined biologically since it is a property independent of the stability of the dissolved substance in the oil.
3. Quinol is not effective in rendering an inferior oil suitable for the biological test.

We wish to express our thanks to Dr F. H. Carr and to Dr S. W. F. Underhill for their constant help and encouragement and to the Directors of The British Drug Houses, Ltd., for allowing us to publish this work.

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CCXXII. EMULSIN IN CERTAIN MARINE INVERTEBRATES AND MICRO-ORGANISMS.

By DENIS LLEWELLYN FOX.

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(*Received July 31st, 1934.*)

According to Giaja [1906] emulsin had not been reported in marine animals prior to the time of his researches on the subject. He investigated a number of molluscs, demonstrating amygdaloclastic emulsin in the intestinal tracts of the gastropods: *Aplysia punctata*, *Patella vulgata*, *Trochus turbinata*, *Buccinum undatum*, *Doris tuberculata* and *Haliotis tuberculata*; in the lamellibranchs: *Tapes desiccata*, *Pecten maximus*, *Mya arenaria* and *Mytilus edulis*. Neither the cephalopods nor any of the fishes that he examined showed the presence of emulsin, whereas the echinoderms *Asterias glacialis* and *Echinus acutus* were shown to possess it.

Later investigations by the same author [1907] showed that the alimentary tract of several genera of marine crustacea and one fresh-water genus possessed the ability to hydrolyse amygdalin and a number of other glucosides. Bierry and Giaja [1906] had demonstrated the presence of emulsin and lactase in the land gastropods *Helix*, *Limax*, *Lymnaea* and *Planorbis*, and Giaja [1910] carried out further studies on the capacity of the digestive juice of the edible snail, *Helix pomatia*, to hydrolyse amygdalin with the production of hydrocyanic acid.

While it has long been known [Buchanan and Fulmer, 1930] that many terrestrial, fresh-water and intestinal bacteria, as well as a number of yeasts and moulds, are able to utilise amygdalin in their nutrition, some having been shown to do so with the production of hydrogen cyanide, it has not been previously recorded, to the writer's knowledge, that many marine bacteria and kindred micro-organisms possess this property in sea-water nutritive media containing amygdalin.

The desirability of investigating a number of micro-organisms with regard to this property was brought out especially with reference to the possibility of their playing some rôle in digestive processes in the alimentary tracts of marine animals.

For the detection of HCN Guignard's test [see Haas and Hill, 1932] and the silver iodide test developed in this laboratory [Fox, 1934] were used. For quantitative determination of HCN produced, the aeration method used by Roe [1924] and by Bishop [1927] was employed.

Toluene-preserved aqueous extracts of either the whole alimentary tract or of the crystalline style alone[1] of the large California sea mussel, *Mytilus californianus*, produced free HCN from amygdalin substrates at room and at blood temperatures. Heating to boiling completely inactivated the enzyme. Living

[1] Yonge [1926] demonstrated an amygdaloclastic enzyme in the digestive diverticulum of the oyster, *Ostrea edulis*, but detected no such enzyme in the style of this mollusc.

mussels (two) kept in an aerated solution of 0·1 % amygdalin in sea-water produced in 43 hours about eight times as much HCN as did naturally-occurring mixed marine micro-organisms in the same volume of sea-water in the absence of the animals (*i.e.* 0·00044 as contrasted with 0·000054 g. HCN respectively). There was always a lag in the inception of amygdaloclastic action by mussel-emulsin in both the living and killed animals. In the presence of amygdalin, living mussels excreted the enzyme into the surrounding water. Here it retained its capacity to hydrolyse amygdalin after the removal of the animals and the preservation of the solution with toluene. In no case did amygdaloclastic micro-organisms, killed with this preservative or with chloroform, produce HCN from the glucoside.

Sixty-nine different species of bacteria, six actinomyces, three moulds and two yeasts (all isolated from the ocean in Dr C. E. ZoBell's laboratory, as yet unidentified as to species) were investigated.

(It should be stated here that the existence of typical marine moulds and actinomyces is still a moot question among bacteriologists and mycologists, but certain micro-organisms, indistinguishable from these respective groups, were isolated from the ocean here and will be correspondingly referred to in this paper.)

The *yeasts* and *moulds* were inoculated in duplicate from pure cultures on to sea-water agar slants containing nutritive materials and 0·1 % amygdalin[1]. Controls of (1) each fungus without amygdalin in otherwise identical culture medium and (2) sterile amygdalin-containing culture slants were set up in each case. The tubes were incubated at 30°. In from four to ten days, HCN was produced by these fungi, excepting one of the two yeasts. Controls of both types were negative.

The six *actinomyces* were inoculated in duplicate from pure cultures into nutrient sea-water broth containing 0·1 % amygdalin, with the two kinds of parallel controls described above. The cultures were kept in the laboratory in diffused light, at room temperature, for 96 days.

While all of them multiplied rapidly and all eventually produced HCN in both tubes (all controls remaining negative) the interval preceding cyanogenesis was usually a long one (10–30 days).

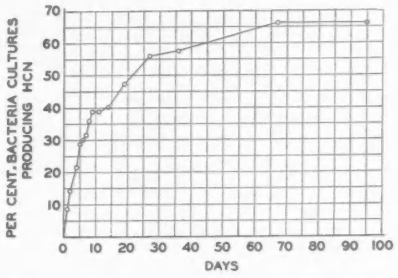

Fig. 1. Numbers of isolated marine bacteria producing HCN from amygdalin, plotted against the passage of time in days.

Each culture of *bacteria* was prepared in duplicate in the same kind of sea-water medium as were the actinomyces and was maintained under identical conditions, with similar controls. The curve (Fig. 1) shows the course of HCN

[1] Coleman and Bell, C.P.

production by increasing numbers of cultures up to a total of forty-six with the passage of time in days.

The control cultures in all the experiments with micro-organisms (*i.e.* tubes containing organisms without amygdalin and those containing sterile solutions of the amygdalin) produced no HCN during the entire experiment.

The virtually complete agreement of both duplicates (only one exception in the case of a very late HCN-producer) and the fact that almost all cases of HCN production manifested themselves simultaneously in both duplicates, added to the fact that the amygdalin control tubes remained sterile, would lead one to conclude safely that all or the great majority of the cultures remained free from contaminants.

The tardiness of HCN production in a number of duplicate amygdalin cultures might indicate several interesting possibilities. One that has been suggested is that certain associated organisms of the dormant commensal type may spring up when the other strain has completed all or a part of its life cycle, and that these organisms differ from the others in physiological characteristics; another is the conceivable acquisition of new physiological properties with gradual adaptation to slowly increasing osmotic pressures attending the evaporation of water from the medium through the cotton plugs; and still another is the changing of metabolic habits with age, or with certain stages in the life cycle.

The experiments demonstrate that the California mussel can produce free HCN from amygdalin independently of the presence of micro-organisms and that the majority of marine bacteria and kindred micro-organisms (such of these as are capable of living and multiplying in sea-water) that were examined are capable of producing free hydrocyanic acid by destruction of amygdalin.

It was demonstrated by Emerson *et al.* [1913], and by Clawson and Young [1913], that pure cultures of *B. fluorescens*, *B. violaceus*, several strains of *B. pyocyaneus* and other bacteria described, but not named, produce HCN from a number of different protein substrates.

While the micro-organisms studied in this laboratory did not produce HCN from broth in the absence of amygdalin, and while certain forms produced it from the glucoside rather promptly, it cannot be stated with certainty whether HCN production was primarily the result of hydrolysis or of oxidation.

Since this physiological property is possessed by so many of the marine micro-organisms and by a number of herbivorous invertebrates, it seemed to be of interest to examine some plants of the ocean for the presence of amygdalin. This has been done without a single positive result.

Flourishing cultures of each of the yeasts, moulds, actinomyces and bacteria, when killed with heat, toluene or chloroform, failed to evolve HCN after the addition of a highly active emulsin, even after a long period of contact. Macerated leaves of the common eel-grass, *Zostera marina*, and crushed blades of the giant kelp, *Macrocystis*, and of the "feather-boa," *Egregia*, also gave negative results.

The capacity of living marine organisms to break down amygdalin and live in the presence of free HCN is interesting, but the detection of amygdalin or other cyanogenetic glucosides in marine plants which might serve as food for such organisms would render the above fact of considerable significance regarding certain aspects of nutrition and a part of the nitrogen cycle of the sea.

SUMMARY.

1. Living mussels (*Mytilus californianus*), or toluene-preserved extracts of either their viscera or the crystalline style alone, can bring about the hydrolysis of amygdalin with the production of free hydrocyanic acid.

2. In the presence of amygdalin, the mussel discharges into the water the enzyme which breaks down the glucoside.

3. Three marine moulds, one of two marine yeasts, six actinomyces from the ocean and forty-six out of sixty-nine bacteria from the same source produced hydrocyanic acid from amygdalin after intervals of various durations.

4. None of the amygdaloclastic micro-organisms retained the cyanogenetic property when killed with toluene or chloroform.

5. None of the micro-organisms produced HCN from the peptone control cultures.

6. No source of amygdalin in marine plants, including all the micro-organisms studied, a marine flowering plant and some kelps, has so far been detected in this laboratory.

It is a pleasure to acknowledge the aid of Dr C. E. ZoBell, Mrs C. B. Feltham and Mrs T. Wells, of the Section of Microbiology of this Institution, for supplying me with cultures of the different marine micro-organisms studied and for giving helpful advice.

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CCXXIII. THE INFLUENCE OF DIFFERENT CATIONS ON THE GROWTH OF YEAST CELLS.

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(*Received August 1st, 1934.*)

The results of previous work, especially that of Loeb and other investigators on marine organisms, have shown the important influence which electrolytes exert on cell growth. Broadly speaking these researches indicate that the influence of the ions in physiological concentration agrees with their position in the Hofmeister series; in other words, it runs parallel with the influence that the ions probably exert on the imbibition of the cell colloids. Ions which favour imbibition, in general, aid growth.

The cations would seem to be of special importance; they apparently play a much more important part in natural cell processes than the anions. It has been frequently demonstrated that as regards potassium there is a direct relation between spontaneous cell growth and potassium content of the tissue. Thus researches on tumours have shown that potassium content and the intensity of cell growth, in general, run parallel. We are thus led to the conclusion that potassium is a natural activator of cell growth. Also it has been shown in recent investigations on animal tissue (chiefly tumours) that potassium increases energy metabolism [see Lasnitzki, 1934]. Energy metabolism (respiration and fermentation) must, however, be considered as the source of that energy which renders possible processes of growth. Thus we are forced to the conclusion that the influence of potassium on growth takes place, wholly or in part, through its influence on the energy metabolism of the growing cell.

While potassium is considered as a natural activator of cell growth its influence, in all its aspects, might be only a special example of a general rule. In this respect it may be asked, therefore, whether not only the growth but also the energy metabolism of the cell may not be subjected to such a general rule. One would expect that the various ions, provided they could enter the cell, would influence cell metabolism and growth in the same direction and to the same degree. We have undertaken comparative researches on this question, especially as regards the group of alkali cations of which the most important representative is potassium.

For investigations of this nature it is necessary to carry out quantitative experiments on growth and energy metabolism on one and the same material and under the best possible parallel conditions. For this purpose we found yeast to be the most suitable material. As regards energy metabolism we limited ourselves to measurements of the alcoholic fermentation. These investigations will, however, be published in a later communication. In the present paper we shall deal only

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with the amount of growth obtained. In the first place we investigated the influence of alkali cations: lithium, sodium, potassium, rubidium and caesium. In addition we also investigated the influence of magnesium and calcium.

Such investigations seemed to us to be necessary in view of the fact that, so far as we are aware, very little systematic research in this direction has been performed. Mitra [1917] investigated the influence of potassium, sodium, calcium and magnesium chloride on the growth of wine yeast. He found that all four salts in a weak concentration accelerated growth, although to a varying degree, while in a stronger concentration they, on the whole, hindered it, again to a varying degree. On mixing two salts there was frequently a definite antagonistic effect. Speakman *et al.* [1928] investigated the influence of sodium chloride on the growth of brewer's yeast. Mitra worked with a synthetic culture medium while these authors grew their yeast on wort. They found that the stronger the concentration of sodium chloride which was added the more was the growth of the yeast hindered.

Technique.

In our researches we used ordinary baker's yeast, and estimated growth volumetrically. We used as culture medium a synthetic medium of which the basis was "Laurent salt solution" [see Koch, 1922]. In view of the absence of specific growth factors vigorous growth could not be expected. The medium, which was fairly acid in reaction to inhibit the growth of bacteria, was prepared by adding together two stock solutions, the standard "suspension fluid" in which the yeast was homogeneously distributed and the variable "addition solution." All stock solutions were conserved sterile, separately on ice. For special details as to concentration *etc.* see later.

We proceeded as follows. A small, accurately weighed amount of fresh yeast was shaken for about 10 minutes with 30–40 ml. of suspension fluid so as to obtain a homogeneous cell suspension. This was centrifuged at a high speed for about five minutes, the supernatant fluid poured off and the solid material diluted with the suspension fluid so as to correspond to 15 mg. of fresh yeast per ml. This cell suspension was distributed, with continuous shaking, in tubes of the form shown in Fig. 1, 2 ml. being placed in each tube. The tube consists of an upper wide, and a lower narrow part, the latter having a capacity of a little over 0·1 ml. and being graduated into 20 parts of 5 μl. each (not shown in figure). The distance between two graduated marks is great enough easily to allow a reading to 1 μl. and possibly to 0·5 μl. The yeast suspension is added in such a way that the under part of the tube is at first empty (Fig. 1, *a*). We then added to each tube 1 ml. of the addition solution[1], and as control 1 ml. of distilled water. Each tube was duplicated. The tubes were then closed with a cellulose plug and left at room temperature for 21 to 24 hours. A longer time than this was unnecessary since growth of the yeast certainly ceases before this. In order to avoid a large drop in temperature during the night the tubes were placed in a wide-necked thermos flask. Our temperature measurements in the whole of the experiments showed maximum oscillations between 20 and 25°.

Fig. 1.

[1] As a result of special tests it appears that there is no difference if the yeast cells are placed at once in contact with the cation to be tested, or (as in the present case) if the latter is only subsequently added to the yeast suspension.

At the end of the experiments a few drops of 40 % formaldehyde solution were added in order to avoid bubbles of gas from fermentation of the yeast, and the narrow under part of the tube was filled with fluid by removal of the air with a fine capillary tube. The tubes were then centrifuged at a high speed for five minutes, whereby the whole of the yeast was concentrated in the narrow part (Fig. 1, *b*). The volume of the yeast column was then immediately estimated, and the mean value of the two estimations taken. The difference between the two parallel estimations was, as a rule, between 0 and 3 μl. Similarly the volume of yeast at the beginning of the experiment was estimated, with usually two control estimations.

Results.

1. *Alkali cations.*

The suspension fluid for these investigations had the following composition:

Diammonium hydrogen phosphate, $(NH_4)_2HPO_4$ ...	0·1 g.
Magnesium sulphate, $MgSO_4$, $7H_2O$	0·03 g.
Glucose	7·5 g.[1]
Asparagine	0·75 g.[1]
Tartaric acid	0·15 g.
Dist. water	*ad* 100 ml.

We utilised as addition solutions 0·03 *M* solutions of lithium, sodium, potassium, rubidium and caesium chlorides. The concentration of alkali chloride after the mixing in the proportion of 2 : 1 was 0·01 *M*, and the concentration of magnesium sulphate ($MgSO_4$) 0·008 *M*. The p_H of the control medium was about 3·1.

The results of all the experiments are given in Fig. 2; the results of individual experiments being also shown in Table I. It is seen that in the

Table I.

Experiment no.	Date (1932)	Duration in hours	Original volume of yeast in μl.	Volume of yeast in μl. at end of experiments					
					Addition of				
				Control	LiCl	NaCl	KCl	RbCl	CsCl
1	26. v	21	30	36	40	49	69	63	48
1 *a*	2. vi	21	(37)	(43)	—	—	(69)	—	—
2	9. vi	24	31	39	40	47	70	70	48
3	13. vi	22	29	30	37	54	68	68	48
4	16. vi	22	29	36	37	52	67	69	48
5	20. vi	23	31	39	38	55	69	66	47
6	23. vi	23	28	35	38	48	62	67	46
7	5. ix	22	26	33	31	40	59	53	38
8	7. ix	22	29	31	34	51	67	69	43
9	7. xi	23	26	36	36	53	67	58	43
10	9. xi	23	27	31	39	52	65	70	48
	Mean		29	35	37	50	66	65	46
	Mean increase			6	8	21	37	36	17

control nutrient solution there is only a slight increase of yeast cells. The increase is about 20 %. On the other hand we see that all five alkali chlorides favour growth. Individually, however, their actions are very different, and we can

[1] As was found in a preliminary experiment with addition of potassium chloride, doubling the concentration of glucose or asparagine had no influence on the result.

separate them into three groups. The first group is represented by lithium chloride, and here the influence, if any, is very slight. Compared with the control solution there is a doubtful increase of only about one-third. The second group, represented by caesium and sodium chlorides, has a medium influence. Caesium chloride gives an increase of about 60 %, and sodium chloride over 70 %. The yeast increases in the caesium solution almost three times and in the sodium solution about three and a half times as much as in the control solution. The third group, represented by rubidium and potassium chlorides, has the greatest influence. The yeast increase amounts in both to about 125 %. The increase is about six times as much as in the control solution.

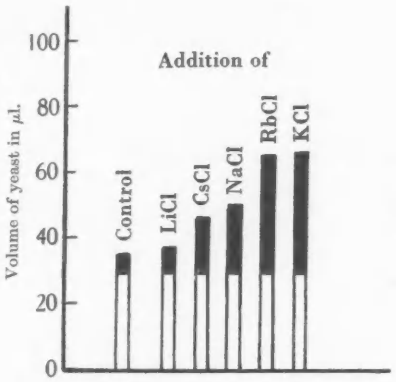

Fig. 2. Original volume of the yeast: *white*. Increase of volume: *black*.

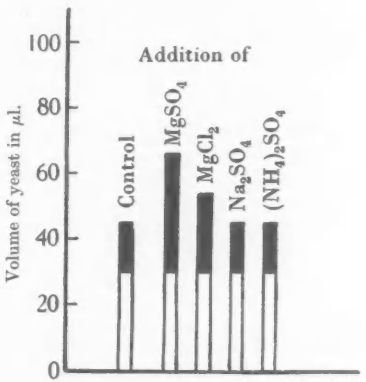

Fig. 3. Original volume of the yeast: *white*. Increase of volume: *black*.

As the chlorine ion is a common constituent of the addition solutions we must ascribe the difference in action to the influence of the alkali cations. We see that, as a whole, they favour the growth of the yeast, and that their actions correspond with their position in the Hofmeister series of cations. It may be remarked that on microscopical examination the yeast cultivated in the several different media shows no definite morphological differences.

In five experiments (Nos. 3–7) the influence of 0·01 *M* ammonium chloride was also investigated. The average values were:

At the beginning	29
At the end, control	35
At the end with addition of NH_4Cl ...	33

(The values found in order were: 31, 32, 36, 35, 31.)

The addition of ammonium chloride to the nutrient medium, already containing ammonium ions, had thus no growth-promoting influence.

2. *Magnesium.*

In these experiments the suspension fluid contained 0·015 *M* KCl and was magnesium-free. Otherwise the composition was similar to that of the previous experiments. The addition of magnesium was made as magnesium sulphate and magnesium chloride. Further parallel estimations with the addition of sodium

and ammonium sulphates were made in order to test the influence of the SO_4 ions. The concentration of the addition solutions was 0·024 *M* in 9 experiments, while in 4 additional experiments performed previously it was $2\frac{1}{2}$ times greater. On mixing in the proportion of 2 : 1 the concentration of the salts added was 0·008 and 0·02 *M* respectively; and the concentration of potassium chloride, as in the previous experiments, 0·01 *M*. The p_H of the control solution was about 3·4.

Table II.

Experiment no.	Date (1932)	Duration in hours	Original volume of yeast in μl.	Volume of yeast in μl. at end of experiments				
					Addition of 0·008 *M*			
				Control	$MgSO_4$	$MgCl_2$	Na_2SO_4	$(NH_4)_2SO_4$
1	13. x	22	31	46	53	50	48	45
2	17. x	22	30	43	59	53	44	40
3	24. x	22	31	48	71	58	54	56
4	26. x	23	28	43	71	56	41	41
5	1. xi	23	30	40	72	53	41	37
6	11. xi	23	31	45	70	59	46	47
7	18. xi	23	31	52	66	54	—	52
8	28. xi	21	30	46	58	53	44	—
9	6. xii	21	29	42	73	53	43	—
	Mean		30	45	66	54	45	45
	Mean increase			15	36	24	15	15
					0·02 *M*			
10	10. v	24	32	47	63	53	49	—
11	20. v	24	31	54	64	58	50	—
12	25. vii	22	32	51	76	58	49	50
13	29. vii	22	33	49	69	54	44	48
	Mean		32	50	68	56	48	49
	Mean increase			18	36	24	16	17

The total results of the 9 experiments with the weak concentration of added salts are given in Fig. 3, the results of individual experiments being shown in Table II, Nos. 1–9. In the control solution the increase in yeast was 50 %. The addition of $MgSO_4$ gave an increase of 120 %. This figure corresponds with that of the previous experiments with the same medium (addition of potassium), while the larger figure obtained in the control solution shows that the presence of potassium chloride alone in the medium is more effective than the presence of magnesium sulphate alone. If magnesium sulphate is replaced by magnesium chloride growth-promotion is less. The yeast increase amounts to only 80 %. If magnesium sulphate is replaced by sodium or ammonium sulphate growth is similar to that in the control solution.

In the 4 other experiments with the $2\frac{1}{2}$ times increase in the concentration of the salts we obtained about the same figures (Table II, Nos. 10–13). Therefore it appears that the actions of both magnesium salts are within wide limits independent of their concentrations, and also that sodium and ammonium sulphates remain inactive even when the concentration is augmented.

That magnesium sulphate increases growth more than magnesium chloride cannot be ascribed to a growth-promoting action of the SO_4 ions, in view of the inactivity of sodium and ammonium sulphates. Also a reversal action, which might occur with the increase of Cl ion concentration from 0·01 to 0·026 g.-ion/l., probably does not enter into the question because the further increase to

0·05 g.-ion/l. is without influence[1]. Therefore it probably depends upon the SO_4 ions favouring the action of the magnesium without themselves exerting any influence on growth. As a possible explanation of this action, it may be that the ability of the yeast to absorb the magnesium depends upon the nature of the accompanying anions. If this is so SO_4 ions are more favourable to the absorption of magnesium than are Cl ions.

3. *Calcium.*

In Exps. Nos. 1–7, Table II, we also investigated the influence of 0·008 *M* calcium chloride, in order to test if, and in what degree, magnesium can be replaced in its growth-promoting activity by calcium. The average figures were:

At the beginning	30
At the end, control	45
At the end with addition of $MgCl_2$ ...	55
At the end with addition of $CaCl_2$ ...	47

(The values found in order were: 47, 45, 53, 46, 51, 44, 46.)

From these results we must conclude that magnesium is not, or at the most only to a slight degree, replaceable by calcium. How far the difference in the action of these two bivalent cations is related to their positions in the Hofmeister series must meanwhile remain an open question.

Summary.

The influence of different cations on the growth of baker's yeast has been investigated. The yeast was cultivated in a synthetic medium. For the investigation of the alkali cations it contained magnesium sulphate as a constant constituent. All alkali cations favour growth of the yeast. They differ among themselves, however, their actions agreeing with their positions in the Hofmeister series. Lithium increases growth very slightly, if at all, potassium and rubidium strongly, while sodium and caesium have an intermediate effect. Magnesium also exerts a growth-promoting influence, these investigations being carried out in a medium containing potassium. Magnesium sulphate acts more strongly than magnesium chloride, although this difference in all probability cannot be ascribed to a direct influence of the anions. Magnesium cannot, or at the most can only to a slight degree, be replaced by calcium.

[1] This does not exclude the possibility that addition of Cl ions as such might have a slight growth-hindering influence (see the experiments with ammonium chloride).

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CCXXIV. INVESTIGATIONS ON THE NATURE OF HAEMOPOIETIN, THE ANTI-ANAEMIC SUBSTANCE IN HOG'S STOMACH.

II. THE PRODUCTION OF A THERMOSTABLE HAEMOPOIETICALLY ACTIVE SUBSTANCE SIMILAR TO OR IDENTICAL WITH THE ANTI-ANAEMIC PRINCIPLE OF LIVER BY THE ACTION OF THE THERMOLABILE HAEMOPOIETIN ON BEEF.

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(*Received August 4th, 1934.*)

In Part I of this series [Klein and Wilkinson, 1933] we reported the preparation and properties of concentrates containing haemopoietin, the anti-anaemic substance in hog's stomach, and these experiments seemed to suggest that haemopoietin had different properties from the active anti-anaemic principle present in liver and was probably enzyme-like in its nature. Further work has confirmed our suggestion that at least two different anti-anaemic factors exist; one, a thermolabile substance, haemopoietin, present in hog's stomach and not as yet separable from the proteins; the other, a thermostable protein-free substance found in liver. The relationship between these two substances, which are so different chemically but nevertheless apparently similar in their clinical effects on cases of pernicious anaemia, is of considerable interest.

This question has already been mentioned in a preliminary report published elsewhere [Wilkinson and Klein, 1933]. The experiments there described confirm our views as to the enzymic nature of haemopoietin and demonstrate the formation of a substance very similar to, if not identical with, the active principle of liver *in vitro* without the use of liver by the action of concentrates of haemopoietin on beef. This change has hitherto been believed to take place only *in vivo*. In the present paper we describe further experiments along these lines and have indicated the possible relationship between the two anti-anaemic principles.

EXPERIMENTAL.

Methods of testing haemopoietic activity.

As we have previously indicated, no chemical or reliable biological tests are known for assaying the haemopoietic value of preparations used for the treatment of pernicious anaemia, and the only available method involves clinical trial on persons suffering from this disease.

The details of the method have previously been described elsewhere [Wilkinson, 1933, 2; Klein and Wilkinson, 1933] but it appears necessary to emphasise here once again the importance of choosing very carefully and adequately controlling all the clinical material used in these tests.

Since liver preparations can be freed from proteins and blood-pressure depressants without impairing their haemopoietic activity, it is possible to administer them not only orally but also by intramuscular or intravenous injection. The latter parenteral route is a far better method, since the active principle thereby finds its way into the blood-stream very quickly, while there is no loss through lack of absorption or destruction in the alimentary tract; consequently, there is a much more rapid response to the treatment. Stomach preparations containing haemopoietin, on the other hand, cannot be given parenterally but only by mouth, since it has not yet been found possible to remove proteins from them without removing the associated haemopoietin.

Experiments with hog's stomach fractions.

Fractionation of press juice by isoelectric precipitation.

In a typical experiment, 1 litre of press juice obtained from the mucosa of hog's stomach [Klein and Wilkinson, 1933] was brought to p_H 4·2 by the careful addition of *N* HCl and the mixture filtered through a series of No. 41 Whatman papers. The precipitate, fraction J 1 (mucosa), was washed with absolute alcohol and dried over sulphuric acid in vacuum-desiccators. The yield was about 8–9 g.

The filtrates from fraction J 1 (mucosa) were adjusted to p_H 6·2 and precipitated by stirring into 4 volumes of 90 % alcohol cooled to 0°. The precipitate of fraction J 2 (mucosa) was collected, washed with absolute alcohol and dried over sulphuric acid in vacuum-desiccators. The yield was about 9–10 g.

Properties of fraction J 1 (mucosa). Fraction J 1 (mucosa) was a pale yellowish-brown powder giving all the usual protein reactions. It contained 11·4 % N, 1·3 % P and 4·5 % ash. The ash gave a very strong phosphate reaction and contained also traces of sodium, calcium and sulphate. Fraction J 1 (mucosa) was extremely active peptically (*cf.* Table I) but contained no haemopoietin when tested clinically.

Properties of fraction J 2 (mucosa). Fraction J 2 (mucosa) was a very pale buff powder almost completely soluble in water giving all the usual protein reactions. It contained 12·5 % N, 2·4 % P and 10·0 % ash. The ash contained sodium, potassium, calcium, iron, magnesium and phosphate, and a trace of sulphate. Fraction J 2 (mucosa) had only moderate peptic activity (*cf.* Table I) and was only moderately active in the treatment of pernicious anaemia. Thus, case 390, receiving 5 g. daily, gave the following blood picture: Maximum reticulocyte response, 10·8 % on the eighth day of treatment. First day of treatment: red cells, 1,220,000; haemoglobin, 34 %; colour index, 1·4. Thirteenth day: red cells, 1,750,000; haemoglobin, 54 %; colour index, 1·6.

Properties of various stomach fractions.

In previous communications [Wilkinson and Klein, 1932; Klein and Wilkinson, 1933], the preparation of concentrates of haemopoietin from the press juice of hog's stomach was described. The properties of some of these fractions, in so far as they have any bearing on later experiments in this paper, are summarised in Table I.

Table I. *Properties of stomach fractions.*

Fraction	Method of preparation	Peptic activity	Time taken to digest egg-albumin mins.	Haemopoietic activity	Daily dose to case of P.A. g.
P 5	Precipitation of press juice from whole hog's stomach with alcohol	+	7	+	5
P 5 (mucosa)	Precipitation of press juice from mucosa of hog's stomach with alcohol	+	4–5	+	5
P 5 (i)	Dissolution of fraction P 5 in $N/10$ HCl and precipitation of addition by NaOH to p_H 4·2	+	4–5	–	7·5
P 5 (ii)	Filtrate from fraction P 5 (i) brought to p_H 6·2 and precipitated with alcohol	Slight	40	+	5
J 1 (mucosa)	Precipitation of press juice from mucosa of hog's stomach by bringing to p_H 4·2 with HCl	+	5–6	–	5
J 2 (mucosa)	Filtrate from fraction J 1 (mucosa) brought to p_H 6·2 and precipitated with alcohol	Moderate	15	Moderate	5
Commercial scale pepsin	Treatment of hog's stomach by the usual commercial methods	+	2	–	60

Inactivation of haemopoietin by heat.

5 g. of fraction P 5 were ground up with 100 ml. of water, and the mixture was heated to 60–65° for half an hour with constant stirring on the water-bath. The resulting product was found to have lost all its peptic activity, and daily administration to cases of pernicious anaemia indicated that it no longer possessed any haemopoietic activity, since no reticulocyte crisis or improvements in the blood counts were noted.

Incubation of fraction P 5 with water.

5 g. of fraction P 5 were ground up with 150 ml. of water, and the mixture was incubated for 6 hours at 37°. The product was then heated to 60–65° for half an hour to inactivate haemopoietin, cooled and given daily by mouth to each of three cases of pernicious anaemia. A small reticulocyte peak (12·4 %) was observed in one patient and may have been due to a spontaneous remission; two other cases failed to show any responses to this treatment, consequently the haemopoietic activity of the product must have been almost negligible.

INCUBATION OF STOMACH FRACTIONS WITH BEEF.

The general procedure was as follows. The experimental stomach fraction was added to minced lean beef-steak and water and after thorough mixing brought to a temperature of 37° by careful heating on the water-bath and finally incubated at that temperature with occasional stirring. All the incubation mixtures had a slightly acid reaction (p_H 5·7) which remained unaltered during the incubation.

The subsequent procedure was based on the fact that haemopoietin is inactivated by exposure to a temperature of 60–65° for half an hour, whereas the active principle of liver is thermostable under these conditions.

A. Oral experiments.

Incubation of beef with fraction P 5. A mixture of beef (100 g.), fraction P 5 (5 g.) and water (120 ml.) was incubated for 2 hours at 37°, heated to 60–65° for half an hour and cooled. The product given daily by mouth to cases of pernicious anaemia was found to be active. Even better responses were obtained in another experiment using 150 g. beef, 5 g. fraction P 5 and 200 ml. water when the incubation time was increased to 6 hours. In this case, the product was filtered and the clear liquid alone administered orally. For example, the effect on the blood picture of case 357 was as follows: Maximum reticulocyte response, 22·4% on the ninth day of treatment. Initial count: red cells, 1,440,000; haemoglobin, 35 %; colour index, 1·2. Twenty-second day; red cells, 2,440,000; haemoglobin, 64 %; colour index, 1·3.

Incubation of beef with fraction P 5 (ii). Similar haemopoietically active products were obtained using a mixture of 100 g. beef, 2 g. fraction P 5 (ii) and 120 ml. water.

Effect of heating fractions P 5 and P 5 (mucosa) before incubation with beef. A mixture of fraction P 5 (5 g.) and water (200 ml.) was heated to 60–65° for half an hour and then incubated for 6 hours with 150 g. beef. The strained juice given daily by mouth to cases of pernicious anaemia produced no haemopoietic responses.

A similar negative result was obtained using fraction P 5 (mucosa).

These experiments confirm the result obtained in a previous experiment that the haemopoietin present in these stomach fractions is inactivated by heating to 60–65° for half an hour.

Incubation of beef with fraction P 5 (i). The material resulting from the incubation of 100 g. beef, 5 g. fraction P 5 (i) and 120 ml. water for 3 hours was given daily by mouth to each of two cases of pernicious anaemia but produced no haemopoietic response. Since fraction P 5 (i) consists largely of pepsin, this experiment confirms our own previous observations and those of Castle *et al.* [1930] that incubation of beef and pepsin yields a haemopoietically inactive product.

B. Injection experiments.

Since a product similar to, or identical with, the active principle of liver appears to be formed in these experiments, the method adopted for the isolation of an active fraction suitable for injection was similar to that used for preparing parenteral liver fractions [*cf.* Wilkinson and Klein, 1934]. It depends upon (*a*) the solubility of the active principle of liver in 70 % alcohol and its insolubility in 95 % alcohol [*cf.* Cohn *et al.* 1930], and (*b*) the solubility of blood-pressure depressants in 95 % alcohol. By utilising these solubility relations, products suitable for intramuscular injection could be easily obtained and they did not possess any marked vasodepressor activity. The products were extremely hygroscopic and were preserved in vacuum-desiccators until required.

Injection product from incubation of fraction P 5 and beef. A mixture of 4 kg. of beef, 80 g. of fraction P 5 and 4 litres of water was incubated at 37° for 6 hours. The product was then heated to 60–65° for half an hour, cooled and filtered at the pump. The clear liquid and washings were treated with sufficient alcohol (about 2½ volumes) to give a concentration of about 70 %, allowed to stand overnight and filtered from the large protein precipitate. The alcoholic filtrates were concentrated *in vacuo* below 50° to about 500 ml., and the last trace of protein was removed by adding absolute alcohol to a concentration of about 70 %, cooling in the refrigerator and filtering. The clear liquid was then

reduced to a thick syrup *in vacuo* below 50° and poured into 10 vols. of absolute alcohol. After stirring until the product was almost solid, the alcohol was replaced by fresh liquid and the stirring continued until the substance became brittle and could be easily broken up and powdered. The pale yellow-brown solid was collected, washed with absolute alcohol and dried and preserved over sulphuric acid in a vacuum-desiccator (yield, 43 g.). This product (S.I. 1) dissolved in the minimum amount of water and sterilised by filtration through a Seitz filter was found to be very active when given by intramuscular injection; *e.g.* case 370 received a total dosage of 43 g. over a period of twelve days. Maximum reticulocyte response, 19·1 % on nineteenth day of treatment. The red cell count rose from 1,160,000 to 2,500,000 and the haemoglobin percentage from 31 % to 74 % without further treatment during 41 days. At the commencement of this treatment the patient was showing a very severe and rapidly relapsing condition—the response was therefore more satisfactory than the figures would appear to suggest.

Discussion.

The results of the experiments described in this paper are summarised in Table II.

Table II. *Responses of cases of pernicious anaemia to various stomach fractions and incubation products.*

Haemopoietically active	Haemopoietically inactive
Fraction P 5 alone	Pepsin alone
Fraction P 5 (mucosa) alone	Fraction P 5 (i) alone
Fraction P 5 (ii) alone	Fraction J 1 (mucosa) alone
Fraction J 2 (mucosa) alone	Beef + water alone
Fraction P 5 or P 5 (mucosa) + beef + water incubated at 37°	Beef + pepsin + water incubated at 37°
Fraction P 5 (ii) + beef + water incubated at 37°	Fraction P 5 + water heated to 60–65° for half an hour
Fraction P 5 + beef + water incubated at 37°, then heated 60–65° for half an hour	Fraction P 5 or P 5 (mucosa) + water heated to 60–65° for half an hour, then incubated with beef at 37°
Fraction P 5 + beef + water incubated at 37°, then heated at 60–65° for half an hour and filtrate worked up for parenteral administration	Fraction P 5 (i) + beef + water incubated at 37°

Inspection of Table II shows that although haemopoietin in the form of fractions P 5, P 5 (mucosa) *etc.*, is active in the treatment of pernicious anaemia, the haemopoietic activity can be destroyed by heating with water to 60–65° for half an hour. Moreover, when the active fractions are heated with water to 60–65° for half an hour they do not yield any active products when subsequently incubated with beef. If, however, any of these unheated concentrates of haemopoietin is incubated with beef and water at 37°, an active product is produced whose activity can no longer be destroyed by heating to 60–65° for half an hour. Thus, during the incubation, the thermolabile haemopoietin produces or is converted into a haemopoietically active product that is now thermostable. Control experiments in which the active fractions were incubated with water alone without beef and subsequently heated to 60–65° gave products of negligible activity. It seems not unreasonable to suppose that the haemopoietin has acted on some constituent in beef and produced another substance similar to or identical

with the relatively thermostable "liver active principle" itself, thus representing a synthesis of the latter from beef and haemopoetin outside the body and without the use of liver. Moreover, the products were worked up by methods similar to those employed for parenteral liver fractions and were found to be active when injected intramuscularly into patients with pernicious anaemia.

The striking resemblance in chemical and other properties between the active principle of liver and the product formed in the above incubation experiments seems to indicate their probable identity and differentiates them sharply from haemopoietin, the active principle of stomach. This is clearly shown in Table III.

Table III. *Comparison of properties of haemopoietin, the active principle of liver and the product formed by the action of haemopoietin on beef.*

Haemopoietin	Active principle of liver	Product formed by incubation of haemopoietin with beef
(1) Precipitated with proteins	Not precipitated with proteins	Not precipitated with proteins
(2) Gives protein reactions	Does not give protein reactions	Does not give protein reactions
(3) Soluble in water	Soluble in water	Soluble in water
(4) Insoluble in 70 % alcohol	Soluble in 70 % alcohol	Soluble in 70 % alcohol
(5) Insoluble in 95 % alcohol	Insoluble in 95 % alcohol	Insoluble in 95 % alcohol
(6) Thermolabile	Thermostable	Thermostable
(7) Gives thermostable product, when incubated with beef	Unchanged when incubated with beef	Unchanged when incubated with beef
(8) Inactivated by acetone *etc.*	Unaffected by acetone	Unaffected by acetone

It seems justifiable then, to assume, that the relationship of haemopoietin to the active principle of liver is that of an enzyme to an end-product and that the latter is formed by enzymic action on some unknown constituent of beef in accordance with the scheme:

Enzyme	Substrate	End-product
Haemopoietin	+ Constituent of beef →	Active principle of liver

Bearing of these results on the work of other investigators.

Many observations recorded not only by ourselves but also by other workers are in harmony with the results just described.

Castle [1929] and his co-workers [1930; 1931; 1932] showed that normal human gastric juice when incubated *in vitro* with the proteins of beef-muscle produced haemopoietically active material, although neither the gastric juice nor the muscle proteins administered separately were active. They suggested, therefore, that there was an "intrinsic factor" of an "enzyme nature" in normal gastric juice which, reacting with the proteins of beef-muscle (the so-called "extrinsic factor"), gave rise to haemopoietically active material. Their evidence, however, was not quite conclusive since they did not record the properties of the active product formed in their experiments or its effect when purified and administered parenterally; but in the light of our own experiments it appears to us justifiable to assume that during incubation of gastric juice and beef enzymic action had taken place similar to that which occurs in our own incubation experiments. In support of this contention, it is interesting to note that one of us [Wilkinson, 1930] has shown that when normal human gastric juice is given with protein food (*e.g.* beef) to patients suffering from pernicious anaemia, a response is obtained, although as Castle also found, the juice is not active when given alone.

Further experiments on the haemopoietic factor in gastric juice have been reported by Morris *et al.* [1932], who have claimed that large amounts of normal human gastric juice after concentration *in vacuo* and neutralisation produced reticulocyte responses when injected into patients having pernicious anaemia. They described the substance in gastric juice (for which they proposed the name *addisin*) as being "thermolabile, dialysable and exhaustible" and concluded that it was probably a hormone. The clinical responses obtained by these workers are, however, open to question and it appears to us that there is not sufficient evidence from their observations to justify their assumption of the existence of yet another anti-anaemic factor [Wilkinson, 1932; 1933, 1]. In this connection, the recent work of Fouts *et al.* [1934] is of great interest. These authors have shown that some change takes place in normal human gastric juice during the process of vacuum-distillation. Fresh gastric juice was subjected to ultrafiltration, but the portions held back by the ultrafilter as well as the ultrafiltrate were inactive haemopoietically. After concentration *in vacuo*, however, the gastric juice gave a response on injection. Moreover, when gastric juice was concentrated by vacuum-distillation and then subjected to ultrafiltration, the portion passing through the ultrafilter was active. In another experiment, gastric juice was subjected to ultrafiltration, and the material held back by the ultrafilter was, after concentration *in vacuo*, found to be active, whereas the ultrafiltrate after concentration *in vacuo* was not active. It is evident, therefore, that the material that could be made active during the process of concentration *in vacuo* was held back by the ultrafilter and was therefore presumably a substance having a large molecular weight, whereas the product formed was a small molecule capable of passing through the ultrafilter. The authors suggest that the haemopoietically active substance is probably formed by action of an "intrinsic factor" upon an "extrinsic factor" in the gastric juice.

These results and their explanation are substantially in agreement with our own work. The enzyme present in gastric juice is probably identical with our haemopoietin and during the prolonged concentration of the juice *in vacuo* acts upon an unknown substrate present in the juice thereby producing the active principle of liver.

The nature of the substrate present in beef (and also in stomach-muscle and apparently in much lesser amount in normal human gastric juice), upon which haemopoietin acts has been the subject of investigation by Castle and his co-workers [1931; 1932] who termed it the "extrinsic factor" and found that it was present in the washed proteins of beef precipitated at p_H 6·0 but not in washed caseinogen or wheat-gluten. It was therefore assumed to be a protein or closely related substance. Castle and Strauss [1932] later found that incubation of normal human gastric juice with autolysed yeast produced haemopoietically active material and they suggested that the "extrinsic factor" was probably vitamin B_2. These conclusions are, however, vitiated by the observations of later workers [Davidson, 1932; Goodall, 1932; Ungley, 1933; Wilkinson and Klein, 1933] that autolysed yeast products (*e.g.* "marmite" or "vegex") taken alone are frequently active when administered to cases of pernicious anaemia. Evidently, the responses obtained by Castle and Strauss might have been due to the autolysed yeast alone. Moreover, the non-identity of the "extrinsic factor" and vitamin B_2 is consistent with the observation of Wills and Naish [1933] that when an extract of egg-white rich in vitamin B_2 is incubated with gastric juice, the resulting material is inactive haemopoietically.

The relationship between the haemopoietic factor in marmite and the active principles of stomach and liver has not yet been satisfactorily elucidated. The

marmite factor, in its general properties (*e.g.* heat-stability, solubility in 70–80 % alcohol, *etc.*) resembles the active principle of liver rather than haemopoietin. Judging by the many therapeutic failures following the use of marmite in the treatment of pernicious anaemia, the active haemopoietic factor must be present in relatively low concentration, and marmite cannot therefore be considered to be a satisfactory substitute for stomach and liver products.

Many workers [Riemann, 1931; Walden and Clowes, 1932; Helmer *et al.*, 1933; Fouts and Zerfas, 1933] have reported that the haemopoietic activity of liver or liver extracts can be markedly increased by incubation with hog's stomach or with normal human gastric juice. These observations all find a ready explanation on the basis of our experiments described in this paper and are in harmony with the view that the enzyme haemopoietin finds its necessary substrate in the fresh gastric or liver tissues.

The results we have obtained appear to have an important bearing on the aetiology and pathology of pernicious anaemia and are consistent with the theory that pernicious anaemia is a deficiency disease characterised by the absence from the gastric mucosa and secretion of a specific enzyme, haemopoietin. It is significant in this connection that pernicious anaemia is almost invariably associated with achylia gastrica and moreover complete or partial removal of the stomach in human subjects has been followed in many cases by an anaemia of the Addisonian type [Wilkinson, 1933, 3].

It is evident that since the reaction

$$\text{haemopoietin} + \text{substrate} \rightarrow \text{active principle of liver}$$

cannot occur in cases of pernicious anaemia owing to the absence of haemopoietin, the livers of such cases must be deficient in the anti-anaemic factor that is present in normal livers. We have recently reported elsewhere the results of experiments bearing on this point [Wilkinson and Klein, 1934] and have shown definitely that whilst normal human livers, like the livers of various mammals and fishes, contain an active anti-anaemic principle, the latter is not present in livers from untreated cases of pernicious anaemia in relapse. If, however, a case of pernicious anaemia is treated with suitable active liver or stomach preparations, the active principle is stored in the liver and consequently the livers of such cases of pernicious anaemia in active remission are found to contain the anti-anaemic liver principle.

Finally, some animal experiments reported by Bence [1933] are also of interest in this connection, for he found that if the stomachs of pigs were completely removed and several months later the animals were killed, the livers were no longer active haemopoietically.

Summary.

1. A relationship of fundamental importance has been shown to exist between the anti-anaemic factors in hog's stomach and in liver.

2. It is found that when concentrates containing the thermolabile haemopoietin (the active anti-anaemic factor in hog's stomach) are incubated *in vitro* with beef-muscle, haemopoietically active material is obtained which is now relatively thermostable. The substance formed, although obtained without the use of liver, appears to resemble the "liver active principle" very closely in its properties, and it can be prepared in a form suitable for intramuscular injection by methods similar to those used for obtaining parenteral liver fractions from liver.

3. A fraction from hog's stomach containing haemopoietin but practically free from pepsin gave similar results when incubated with beef.

4. Pepsin itself, or fractions from hog's stomach shown clinically to be free from haemopoietin, gave negative results when incubated with beef.

5. It is considered that the relationship between the anti-anaemic principles in stomach and in liver is that of an enzyme to an end-product; that is to say, the enzyme haemopoietin requires for its effective action some unknown substrate apparently present in beef, and the result of this enzymic action is a haemopoietically active end-product which is ultimately stored as the active principle in liver until it is required by the body for the production of the red blood corpuscles.

6. This enzymic action which we have been able successfully to imitate outside the body, namely

Unknown substrate in beef + the enzyme haemopoietin → the "liver active principle"

probably takes place in the stomachs of normal individuals but not in cases of pernicious anaemia.

7. Various observations of our own and of other workers are reviewed and shown to be in harmony with the results we have described and the views we have put forward.

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CCXXV. THE NATURE OF THE ACTION OF TYROSINASE.

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Certain oxidising enzymes, such as xanthine oxidase, can bring about oxidation of their substrates even in absence of oxygen, provided some other substance able to function as suitable hydrogen acceptor is present, such as methylene blue. It is probable that such oxidising enzymes do not exert their action on the oxygen or other hydrogen acceptor, but on the substrate and act by activation of hydrogen. Such enzymes Raper [1928] has called anaerobic oxidases. He has designated as aerobic oxidases such oxidising enzymes as have never been observed to bring about their action on their substrate in absence of oxygen, that is to say, the oxygen has not been replaced by any other hydrogen acceptor. It is concluded that such enzymes probably act by activation of oxygen. Enzymes which activate peroxide, such as peroxidase, are classed as aerobic oxidases, because they affect the oxygen potential (as do activators of oxygen) rather than the hydrogen potential of the system.

Tyrosinase is an enzyme which has never been known to exert any effect in absence of oxygen, with the exception of certain observations reported by McCance [1925]. He found that there was slight reduction of methylene blue by catechol or *p*-cresol in presence of glycine, which was considerably hastened by tyrosinase preparations. This he took to indicate that the action of tyrosinase is on the substrate, that it acts by hydrogen activation, and that this is also the method by which it acts aerobically. He found that methylene blue was also slowly reduced by tyrosine, but the reduction was not hastened by tyrosinase. This he attributed to the unsuitability of methylene blue as the hydrogen acceptor for this substrate.

Now it has been established by Raper and his collaborators that the chemical change undergone by a monohydric phenolic substrate such as phenol, *p*-cresol or tyrosine under the influence of tyrosinase in air is the formation of the corresponding catechol derivative, and that this is further oxidised to an *ortho*quinone before subsequent changes occur. It is difficult to understand how a single oxygen atom could be introduced between hydrogen and the nucleus as a result of mere activation of hydrogen. It is evidently necessary to postulate either addition of water to the nucleus, followed by dehydrogenation, or what is more probable, activation of the molecular oxygen. Experimentally, tyrosine has never been shown to be "dehydrogenated" by an inorganic catalyst. In view of the importance of McCance's results therefore an attempt to repeat his essential experiments has been carried out.

Experimental.

When an attempt was made to repeat the essential experiments reported by McCance, difficulty was experienced in eliminating last traces of oxygen. Even an electric pump left enough oxygen to colour *p*-cresol slightly in presence of

tyrosinase. In the present experiments the trace of air left after evacuation was washed out with nitrogen, the process being repeated several times. Nitrogen from the cylinder, however, contains oxygen. This was not removed adequately by passage through several wash-bottles containing alkaline pyrogallol solution, or by passage through a furnace containing reduced copper oxide, for *p*-cresol in a Thunberg tube repeatedly filled with such nitrogen was still slightly coloured by tyrosinase.

Eventually the process of Kautsky and Thiele [1926] for freeing nitrogen from oxygen, adapted as follows, was found to be effective. The apparatus consisted of (1) nitrogen cylinder, pressure gauge and regulator, (2) inverted aspirator containing alkaline sodium hydrosulphite solution, (3) wash-bottle containing 10 % KOH, (4) wash-bottle containing water, (5) 3-way tap, (6) 2-way tap, (7) electric pump and mercury manometer. Into the inlet of the aspirator was fixed a Berkefeld filter to break up the gas into fine bubbles as it entered the liquid. The outlet of the aspirator was covered with a lead disc through which passed glass tubing and was cemented together with white sealing-wax. All the glass ends were joined together with fairly flexible lead (compo) tubing, and the joints were cemented with white sealing-wax. The 2-way tap marked the end of the gas-tight part of the apparatus. All liquids to be used were well boiled to expel air and cooled overnight in a desiccator over pyrogallol solution into which alkali was spilt after evacuation. (Since *p*-cresol is very volatile in steam, the solution was made stronger than required and was always tested to make sure that plenty remained. To avoid contaminating other liquids, the *p*-cresol must be in a desiccator by itself.)

Before the apparatus was used, nitrogen was run through it for several hours. The substrate solution was placed in a Thunberg tube, the enzyme preparation in a small tube loose inside the Thunberg tube. The cylinder was turned nearly off, and the 3-way tap was turned to cut off the cylinder. The Thunberg tube was then cemented with white sealing-wax through lead tubing on to the 3-way tap. The pump was switched on and the tube evacuated. The 2-way tap was then shut, and the 3-way tap was opened to admit nitrogen. The process was repeated several times. The contents of the Thunberg tube were then allowed to boil to remove last traces of oxygen and to mix the enzyme and substrate. The tube was again filled with nitrogen several times.

The enzyme preparation was freshly prepared for each experiment as follows. Mealworms were fed on cream crackers for some time, then starved for a few days. Of these 5 g. were chloroformed, crushed in a mortar with 20 ml. water, pressed through muslin and centrifuged. The supernatant liquid was discarded, and the residue in the tube was rubbed up with another 20 ml. water and again centrifuged. Four lots of washings were discarded, and the final residue was suspended in 20 ml. water.

The series of tubes was filled as follows: (1) 1 ml. 0·5 % catechol in phosphate buffer p_H 8·0, (2) 1 ml. approximately 0·5 % *p*-cresol similarly, (3) 1 ml. saturated tyrosine similarly, (4) 1 ml. 0·5 % glycine similarly. To each of these tubes was added 1 ml. buffer. (5) 1 ml. catechol solution and 1 ml. glycine solution, (6) 1 ml. *p*-cresol and 1 ml. glycine, (7) 2 ml. buffer. To each tube was added 0·3 ml. *M*/1000 methylene blue in water.

In series I, in addition, 1 ml. water was added to each tube and no enzyme preparation. There was no reduction of the methylene blue in any tube after two days. Series II, as I, but incubated at 80°. Overnight, tubes 3 and 5 were partially reduced. After two days, 5 was completely reduced, 3 considerably, 1 partially; trace of reduction in 4. Series III (tubes 3, 5, 7 only) as II, but with

addition of a little solid thymol to each tube. Overnight, 3 and 5 were partially reduced. After two days, 3 and 5 were completely, 7 partially reduced. Series IV, only 0·5 ml. water was added, and 0·5 ml. enzyme preparation in the little tube. Tubes 4 and 6 were partially reduced overnight, completely after two days, when 3 and 7 were partially reduced. The tubes smelt unpleasantly when opened. Series V, as IV, but with addition of thymol. After two days, very slight reduction in 6 alone—faintly greenish blue—repeated and confirmed. Series VI, as IV, but incubated at 80°. Tube 6 was reduced in 40 mins. and tube 5 in 1½ hours. Overnight 1 and 3 were partially reduced. Series VII, as IV, but incubated at 80° with addition of thymol. After two hours, tubes 5 and 6 were completely reduced. After two days, all tubes were slightly paler. Series VI was repeated at 80° and at 60°, using the same preparation from mealworms. At 80°, tube 6 was reduced in ½ hour, tube 5 in ¾ hour; at 60°, tube 6 in 40 mins., tube 5 in 1 hour.

Table I.

	I	II	III	IV	V	VI	VII
	S	S 80°	S 80° T	S E	S E T	S E 80°	S E 80° T
(1) Catechol	—	+		—	—	+ +	+ +
(2) *p*-Cresol	—	—		—	—	—	+
(3) Tyrosine	—	+ +	+ +	x	—	+ +	+ +
(4) Glycine	—	?+		x	—	—	+
(5) Catechol and glycine	—	+ +	+ +	—	—	+ + + +	+ + + + + approx.
(6) *p*-Cresol and glycine	—	—		x	?+	+ + + + + +	+ + + + + approx.
(7) Buffer	—	—	+	x	—	—	+

S, substrate; 80°, incubated at 80°; T, with addition of a little solid thymol; E, enzyme preparation. ?+, slight reduction after a day or two; +, partial reduction after a day or two; + +, partial reduction overnight; + + + + to + + + + + +, reduction complete in a few hours; x, bacterial development?

The results are summarised in Table I. They indicate that (1) catechol without any enzyme, on heating, slowly brings about a reduction of methylene blue, which is hastened in presence of glycine. This is probably a dehydrogenation of catechol to *ortho*quinone, which oxidises the amino-acid and is reduced back to catechol. The final liquid was brownish pink. In all other instances, reduction resulted in a colourless liquid. This is particularly noteworthy in the case of tyrosine, as it contrasts with the red colour deepening to black produced by the action of tyrosinase on tyrosine in air. (2) The very slow reduction of methylene blue by catechol is slightly hastened by incubation with the enzyme preparation. This may be due to the effect of amino-acids produced by proteolysis of the enzyme preparation, which contains a protease. (3) The slow reduction of methylene blue by tyrosine is not affected by the enzyme preparation. (4) In absence of antiseptic, bacteria probably develop in presence of the enzyme preparation and cause the reduction of methylene blue. (5) Thymol reduces methylene blue, though extremely slowly even at 80°. (6) Even at 80° the reduction of methylene blue by glycine is almost negligible and is not hastened by the enzyme preparation. (7) The reduction of methylene blue by catechol with glycine is very considerably hastened in presence of the enzyme preparation. (8) Reduction of methylene blue is even more rapid by *p*-cresol with glycine in presence of the enzyme preparation.

It is interesting to compare the quickest rate of reduction of methylene blue in these experiments with the oxygen uptake which accompanies the action of tyrosinase on catechol or *p*-cresol. The amount of methylene blue in each

Thunberg tube, 0·3 ml. $M/1000$, is equivalent to 3·36 μl. O_2. The following mixtures as in Series IV, but without methylene blue, were placed in Barcroft micro-respirometers, with buffer in place of substrate solution in the control bottle in each case. Corresponding to tube 1 absorbed 229 μl. O_2 in 15 mins.; to tube 2, 135 μl.; to tube 5, 204 μl.; to tube 6, 212 μl. Oxidation in presence of oxygen is thus of a totally different order of magnitude from the reduction of methylene blue, even when the latter is warmed at 80°.

Precautions were taken to avoid gross contamination with iron by use of freshly glass-distilled water and of phosphate buffer decanted from precipitated iron salt. No coloration whatever was observed on mixing catechol with glycine solution, such as was observed by McCance. The *p*-cresol used had been twice distilled and stored in a paraffin-waxed bottle, as otherwise *p*-cresol is liable to become contaminated with homocatechol.

The enzyme preparation used in these experiments contained no peroxidase, as determined by absence of coloration of guaiacol in presence of hydrogen peroxide.

Discussion.

The essential observations made by McCance have been confirmed. The slight reduction of methylene blue by catechol, particularly in presence of glycine, is considerably hastened by a preparation of tyrosinase from mealworms. The effect of such a preparation in hastening the reduction of methylene blue by *p*-cresol with glycine is even more striking.

While the facts, however, are confirmed, there are certain obstacles in the way of ascribing the phenomenon to the action of tyrosinase.

(1) The definite slight reduction of methylene blue by tyrosine is not hastened by the tyrosinase preparation, although methylene blue evidently is a suitable acceptor for the dehydrogenation of tyrosine, since appreciable reduction of methylene blue is brought about by tyrosine alone. (But possibly some portion of the molecule other than the phenol group is concerned.)

(2) The rate of the oxidation which takes place with methylene blue as hydrogen acceptor is of the order one-hundredth or less of that of the oxidation brought about by the same tyrosinase preparation in air, although methylene blue will usually act as hydrogen acceptor for hydrogen in substrates not sufficiently activated for the hydrogen to be taken up by molecular oxygen, *e.g.* lactic and citric dehydrases.

(3) The optimum temperature for the phenomenon described is about 80°, whereas the optimum temperature for the action of tyrosinase normally is about ordinary temperature.

While there are these difficulties in the way of accepting the phenomenon as due to the tyrosinase in the mealworm preparation, no more satisfactory explanation is at present forthcoming. There is, too, other evidence in support of the suggestion that tyrosinase acts by activation of hydrogen in its substrates.

(*a*) Okuyama [1929] measured the hydrogen potentials developed when tyrosinase was mixed with various substances and concluded, in support of McCance's theory, that it acts by activation of hydrogen in its phenolic substrates, in presence of amino-acid. (The paper, however, contains a discrepancy, between figures 2_2 and 10_2.)

(*b*) The action of tyrosinase on a monohydric phenolic substrate is hastened by hydrogen peroxide (even when the liquid is well aerated with oxygen), or by *ortho*quinone, or by a catechol derivative (which gives rise to hydrogen peroxide and *ortho*quinone). This may be due to the superior oxidation potential of

hydrogen peroxide and of *ortho*quinone over molecular oxygen as hydrogen acceptor [see Pugh, 1930].

(*c*) The same suggestion is supported by the inhibitory effect of substances which might be expected to reduce the oxidation potential. Pugh and Raper [1927] found that benzenesulphinic acid prevented the oxidation of phenol by tyrosinase, while permitting that of catechol. Again, the oxidation of a monohydric phenol is more strongly inhibited by sulphite or by cyanide than that of an *o*-dihydric phenol [Pugh, 1930]. Richter [1934] has shown that *o*-phenylenediamine, bisulphite, potassium iodide and aniline all inhibit the action of the enzyme on monohydric phenols at a concentration at which they still permit action on catechol. He ascribes this to removal of the *ortho*quinone normally produced, which he considers to play a specific part in the reaction; but it may be that the action is essentially one of lowering of the oxidation potential.

The question must be left open. There does, however, appear to be the possibility of co-ordinating all these phenomena by the hypothesis that tyrosinase activates hydrogen in its substrates, and that methylene blue, molecular oxygen, hydrogen peroxide and *ortho*quinone are all, in suitable circumstances, able to act as hydrogen acceptors for the action of tyrosinase.

SUMMARY.

McCance's observation that a preparation from mealworms hastens the reduction of methylene blue by a mixture of catechol or *p*-cresol with glycine has been confirmed. There are obstacles in the way of accepting the suggestion that this denotes the activation of hydrogen in its substrates as the mode of action of tyrosinase, but there is also certain supporting evidence, which is discussed.

I wish to express my thanks to Prof. H. S. Raper for his helpful interest and advice.

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CCXXVI. PIGMENTED MARINE ANIMAL OILS.

I. PIGMENTS FROM THE ANGLER FISH (*LOPHIUS PISCATORIUS*), THE PRAWN (*NEPHROPS NORVEGICUS*) AND THE WHALE.

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(*Received August 4th, 1934.*)

In continuation of a comprehensive investigation into the constituents of marine animal oils [Morton *et al.*, 1931; Heilbron *et al.*, 1931; Castle *et al.*, 1934, *etc.*] we have commenced a study of pigmented specimens, and during the past year have made a preliminary examination of three such oils, *viz.*: angler-liver oil (*Lophius piscatorius*), red whale oil, and the oil of a particular species of prawn (*Nephrops norvegicus*).

The unusual pigmentation of the liver of the angler or monk fish and of the oil extracted from it, was first noted by one of us (J. A. L.). Lovern and Morton [1931] discussed the carotenoid nature of the pigment and its location and development in the liver and examined the absorption spectrum of the oil. Lovern [1931] reported the presence of two pigments, one a neutral substance with carotenoid properties and the other acidic in character. Subsequently he amplified this [1932] and gave an account of the adsorption of the unsaponifiable matter on alumina. We have continued this investigation with a view to the further separation, identification and isolation of the pigments. While our work was in progress Sørensen [1934] published the results of an investigation on the development of the colour in the liver of *Lophius piscatorius* by various treatments and concluded that it was present as a colourless protein-carotenoid complex from which it was liberated on extraction. From the behaviour of the oil and the unsaponifiable material on adsorption on calcium carbonate from carbon disulphide solution and from an examination of the absorption spectra of the carotenoid bands he concluded that the colour was due to the presence of a taraxanthin ester and epi- and hypo-phasic astacene esters [Kuhn and Lederer, 1931, 1]. Our results amplify and confirm those of Sørensen.

Angler fish-liver oil. The pigmented oil was obtained by intimately mixing the minced liver with anhydrous sodium sulphate and extracting with ether. A most striking difference in the pigmentation was observed between the oil obtained by rapidly working up single livers and that yielded by the slower operations involved in attempting to work up large amounts. The first process gives rise to a bright red, and the latter to a dull brown oil in which we have found that extensive destruction of the pigments has occurred. Livers maintained in cold storage still give bright oils, and all such oils retain their colour when kept under nitrogen. In the present communication only bright fresh oils are considered. A solution in chloroform (4·5 %) gave maxima at 518, 483 and 455 $m\mu$, $E^{1\%}_{1\,\mathrm{cm.}}$ 455 = 0·135 [*cf.* Lovern and Morton, 1931]. With antimony trichloride the oil gave a violet colour with a single clear band with maximum at 572 $m\mu$, $E^{1\%}_{1\,\mathrm{cm.}}$ = 0·135, which was not affected by either benzoyl peroxide [Heilbron *et al.*, 1931] or methylindole [Emmerie *et al.*, 1931; Morton, 1932].

Chromatographic analysis of the oil using a 10 % solution in light petroleum

(B.P. 40–60°) and alumina as adsorbent (Merck standardised Brockmann) gave three bands: a rather weak brownish red band (A) near the top of the column, an intense cerise band (B) less strongly adsorbed and a yellow band (C) which passed through the column giving a bright yellow filtrate. Elution by methyl alcohol removed a small portion of (A) and the bulk of (B), the remainder in each case being disengaged by glacial acetic acid. The pigment from the main fraction (B) gave a single broad absorption band with maximum at 497 $m\mu$ in carbon disulphide; the yellow filtrate showed maxima at 468 and 443 $m\mu$ in light petroleum, displaced to 496 and 465 $m\mu$ in carbon disulphide. The intensities of absorption expressed in terms of the original oil were:

$E^{1\%}_{1\text{ cm. (light petroleum)}}$, cerise band (astacene pigment) 0·016;
$E^{1\%}_{1\text{ cm. (light petroleum)}}$, yellow filtrate (xanthophyll pigment), 0·040.

The latter corresponds to approximately 2 mg. xanthophyll per 100 g. oil, the ratio of xanthophyll pigment to astacene pigment being 5 : 2, assuming the same extinction coefficient for the two pigments. The methyl alcohol eluate of band A gives no selective absorption (probably astacene oxidation products), while the pigment removed by glacial acetic acid gave the same absorption spectrum as (B) in carbon disulphide solution.

The pigment from band B re-adsorbed twice on alumina exhibited the typical astacene ester behaviour, being retained at the head of the column [*cf.* Kuhn and Lederer, 1933]. Re-adsorbed on calcium hydroxide from light petroleum, four bands were obtained: (i) at the top, an orange-red band, (ii) a brick-red band, (iii) a violet band and closely associated with it (iv) a very weak brown band. All these exhibited a broad absorption maximum closely resembling that of astacene, no clear difference between the fractions being detectable.

Saponification of (B) with 2*N* methyl alcoholic NaOH yielded a red sodium salt which separated at the interphase on addition of light petroleum. This behaviour is identical with that of the red sodium salt of astacene [Karrer and Benz, 1934]. The free pigment was obtained as a violet-black solid on acidification but the amount was too minute to allow of its further purification. For confirmatory identification we have compared the absorption spectra of the above pigment (465 $m\mu$ in light petroleum) with that of a sample of partially purified crystalline astacene from lobster (463 $m\mu$ in light petroleum, 499 $m\mu$ in carbon disulphide) which is in fair agreement with the value recorded by Sørensen [1934] (503 $m\mu$ in carbon disulphide).

In agreement with this author's statement, we have observed that the spectroscopic values for the yellow band (fraction C) closely resemble those of authentic taraxanthin, giving maxima at 468 and 440 $m\mu$ in light petroleum (taraxanthin, 469 and 443 $m\mu$ in light petroleum). In this connection it is interesting to note that Karrer and Loewe [1934] have recently established the constitution of astacene as 4:5:4′:5′- or 5:6:5′:6′-tetraketo-β-carotene, $C_{40}H_{48}O_4$, while Kuhn and Lederer [1931, 2] have found that taraxanthin is a four-oxygen xanthophyll. The co-existence of these two pigments in the angler oil suggests the possibility that taraxanthin may be the biochemical precursor of astacene in this case, a question which we are at present investigating.

Red whale oil. This material, received by the courtesy of Messrs Lever Bros., Ltd., was obtained from a whale caught in the Antarctic in 1931. Whales giving red body oil are rarely encountered, and except for the fact that their fat is permeated with pigment they appear normal. The condition is regarded as pathological but the present authors know of no detailed evidence concerning this. The oil shows a single broad absorption band with a maximum near 484 $m\mu$ in chloroform, strikingly similar to the characteristic astacene band ($E^{1\%}_{1\text{ cm.}} = 0{\cdot}112$).

The pigment was separated from the bulk of the oil (3000 ml.) by passing the latter in 20–30 % solution in light petroleum (B.P. 40–60°) through a tower packed with fuller's earth admixed with inactive alumina. It was completely held in a narrow zone (2–10 mm.) at the top of the adsorption column and was eluted with methyl alcohol. The oily material was recovered practically quantitatively from the filtrate. By partition between methyl alcohol-light petroleum it proved to be hypophasic (contrasting with the cerise band from the angler oil). The alcoholic phase after concentration (200 ml.) was covered with light petroleum and saponified for twelve hours in the cold with aqueous alcoholic sodium hydroxide (60 ml., 2*N*), a red sodium salt separated at the interphase. Acidification of this salt with acetic acid (50 %) gave a sticky dark red mass, which was washed free from acetic acid and taken up in chloroform. By precipitation of the dried solution with light petroleum a brownish red amorphous solid was obtained. Its absorption spectrum in the visible showed no maximum, the substance being presumably an oxidation product of the pigment. The mother-liquor was evaporated and the minute deep red residue redissolved in light petroleum. This solution exhibited a single broad absorption band near 470 $m\mu$. Addition of aqueous sodium hydroxide reprecipitated the red sodium salt; this was removed, suspended in alcohol (70 %) and acidified with acetic acid (50 %); violet-black needles slowly separated, but the amount was too small for further investigation. From the appearance of these crystals together with the position and general character of the absorption bands of the original oil and of the separated "acid" we conclude that the main pigment of the whale oil is probably a hypophasic astacene ester[1].

Oil from the prawn (Nephrops norvegicus). *Nephrops*, a species of large prawn, differs from the common lobster in that the claws and shell are pink in the natural state and do not change colour on boiling. The oil was prepared by taking the whole animal, mincing, desiccating with anhydrous sodium sulphate and extracting with ether. The pigment is probably derived solely from the shells, as the flesh is white. A chloroform solution of the oil showed a single broad band with maximum near 490 $m\mu$ ($E^{1\%}_{1\,cm.} = 3{\cdot}83$) indicating a pigment content approximately 35 times that of the whale oil.

The original oil (100 ml.) was taken up in methyl alcohol (500 ml.) and cooled to $-10°$; a deep red thick oil separated. On concentration, the mother-liquor deposited flat colourless needles, M.P. 146–147° (acetate M.P. 113–114°) giving no depression in M.P. on admixture with cholesterol. The filtrate on concentrating further provided more heavy red oil, which was combined with the above and the whole adsorbed from a light petroleum solution on alumina. The following complex chromatograph was obtained:

From the top of the column cm.	Band or zone	From the top of the column cm.	Band or zone
3·0	(A) Pink zone	6·8	(C) Sharp red band
3·5	(B) Deep red zone	8·8	— Practically colourless
6·5	— Practically colourless	8·9	(D) Orange band

It is not yet clear whether this series of zones arises from an intrinsic complexity of pigments or whether it is due to interference with the normal process of adsorption by the oily constituents present. Elutions were carried out with methyl alcohol, but in the case of fractions A, B and C a small proportion of pigment remained on the adsorbent and could only be removed by glacial acetic

[1] We are, however, continuing the examination of this pigment, and the corresponding one from the angler, with a view to determining whether the small differences between their spectra and that of pure astacene indicate differences in structure or arise from impurities in the products from the oils.

acid. Band C, which contained the major part of the pigment, was selected for further examination. The eluted fraction was evaporated and the residue crystallised from light petroleum-ethyl alcohol; a small quantity of a colourless solid, M.P. 65–66°, was deposited. This proved to be hentriacontane, $C_{31}H_{64}$, giving no depression in M.P. on admixture with a specimen of the hydrocarbon obtained from *Fucus vesiculosus* [Heilbron *et al.*, 1934]. On concentration of the filtrate a further small crop of cholesterol was obtained. The mother-liquor (3 ml.) was diluted with light petroleum and saponified in the cold for two hours with $2N$ aqueous alcoholic NaOH (5 ml.). The separated red sodium salt was removed, suspended in ethyl alcohol (70 %) and acidified with acetic acid (50 %); the colour of the salt rapidly changed almost to black. The free pigment was removed, washed with light petroleum (B.P. 40–60°) and recrystallised from aqueous pyridine, giving violet-black crystals, M.P. 235° (M.P. pure astacene 240–243° [Kuhn and Lederer, 1933][1].

In solution in pyridine the free compound exhibited a single broad absorption band with a maximum near 488 $m\mu$. A comparison of the absorption curve of pure astacene from lobster, as given by the above authors, with that of our specimen shows the two curves to be very similar, although the maximum recorded by Kuhn and Lederer is near 500 $m\mu$ in pyridine. In carbon disulphide the maximum for our sample lies near 509 $m\mu$, which is in fair agreement with that recorded for the red angler pigment in the same solvent [Sørensen, 1934]. The difference in the melting-points and the location of the absorption maxima suggest that the solid obtained by us was slightly impure. We consider, however, that it is almost certainly astacene.

Summary.

Red oils from the angler fish (*Lophius piscatorius*), the prawn (*Nephrops norvegicus*) and the whale have been examined chromatographically. In each case a violet-black solid, almost certainly identical with astacene, has been isolated.

In the case of the angler-liver oil a yellow pigment is also present, which in solution shows absorption maxima characteristic of taraxanthin [*cf.* Sørensen, 1934]. The occurrence of taraxanthin and astacene together in the angler-liver oil is probably more than coincidence in view of the recent discovery that both pigments contain the same number of carbon and oxygen atoms.

We desire to express our thanks to the Department of Scientific and Industrial Research for a maintenance grant to one of us (E. G. P.) and to Mr A. E. Gillam, who carried out the spectrographic determinations.

[1] Since this work was completed we have seen a paper by Fabre and Lederer [1934, *Bull. Soc. Chim. Biol.* **16**, 105], in which the isolation of astacene (M.P. 228–230°, absorption maximum in CS_2 at 510$m\mu$) from a species of *Nephrops* is reported.

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CCXXVII. ADSORPTION EXPERIMENTS WITH VITAMIN A CONCENTRATES.

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The unsaponifiable matter of halibut-liver oil, after removal of the major part of the sterols, is a viscid orange-brown oil containing 60–70 % vitamin A, which on fractional distillation yields a pale yellow oil [Heilbron *et al.*, 1932]. If the vitamin concentrate, whether purified by distillation or not, be dissolved in light petroleum and passed through a column packed with adsorbent (Al_2O_3 or $Ca(OH)_2$) a red zone appears. Recovery of the adsorbed material by elution of the adsorbent with methyl alcohol shows that the colour is much more intense than that of the original concentrate. In view of the important biological relation between the red pigment carotene and vitamin A, this phenomenon has been studied in detail. At the same time, it was convenient to examine the reported separation of vitamin A into α- and β-forms [Karrer, Walker *et al.*, 1933; Karrer and Morf, 1933; Euler *et al.*, 1934]. As the main distinctions between these two forms and the other fractions obtained depend upon the spectroscopic examination of the colours they produce with antimony trichloride, a brief résumé of the earlier spectroscopic data on this reaction is necessary.

The blue colour characteristic of vitamin A [Carr and Price, 1926] has been shown to exhibit two absorption maxima at 620 and 583 $m\mu$ in concentrates, and 606 and 572 $m\mu$ in oils [Drummond and Morton, 1929; Morton *et al.*, 1931]. Measurements of the intensities of the two absorption bands in the antimony trichloride blue solution for numerous liver oils and concentrates [Gillam and Morton, 1931] showed that the two bands varied so much in relative intensity as to make it seem probable that they were derived from two separate, but related, chromogenic substances. This was the more probable when it was found [Heilbron *et al.*, 1931] that in oils which showed the 572 $m\mu$ band more strongly than the 606 $m\mu$ band, the intensity at 606 $m\mu$ could be increased by oxidation with a little ozone or hydrogen peroxide. Further, the 606 $m\mu$ band could be completely suppressed by adding a few drops of antimony trichloride before the main bulk of the reagent.

Certain indole-like substances likely to be present in liver extracts were found to inhibit the formation of the blue colour [Emmerie *et al.*, 1931]. Thus, a liver oil giving a blue colour with antimony trichloride showing a strong 606 $m\mu$ band and a weak satellite at 572 $m\mu$, when treated with a trace of 7-methylindole before addition of the antimony trichloride, gave a violet colour showing only the 572 $m\mu$ band, the 606 $m\mu$ band having been completely suppressed. These workers [Eekelen *et al.*, 1931; 1932; 1933] regarded this evidence as supporting the theory of two separate chromogens responsible for the two

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absorption bands. The inhibitor substances act in the same way with rich concentrates, suppressing the 617 $m\mu$ band very easily whilst scarcely affecting the 583 $m\mu$ maximum [Morton, 1932].

When richer concentrates of vitamin A ($E^{1\%}_{1\,cm.}$ 328 $m\mu = 1350$) were obtained [Karrer *et al.*, 1931, 1, 2; Heilbron *et al.*, 1932] it was still found that with antimony trichloride the blue colour showed a 617–620 $m\mu$ band and a masked maximum at 583 $m\mu$, the ratio of their intensities being approximately 2 : 1. Later, by a refinement of the distillation method, Carr and Jewell [1933] prepared a still purer product ($E^{1\%}_{1\,cm.}$ 328 $m\mu = 1600$) which with antimony trichloride gave a blue colour showing a clear band at 620 $m\mu$ with only a masked maximum at 583 $m\mu$ (ratio of intensities 5·0 : 2·6). The constant association of these two maxima, even in the purest vitamin preparations, made it seem highly probable that what were originally thought to be two separate chromogens were actually two chromophoric groupings in the same molecule. Karrer and his collaborators [1933; 1934; Euler *et al.*, 1934] have however claimed that, by adsorption on calcium hydroxide, vitamin A concentrates yield two substances, which they suggest correspond to the two chromogens already mentioned. The theory is attractive as it brings vitamin A into line with carotene, where the existence of α- and β-forms is fully established.

Experimental.

After numerous preliminary adsorption experiments we have found that the following experimental conditions yield the best separation of products for a quantitative study.

A distilled concentrate ($E^{1\%}_{1\,cm.}$ 328 $m\mu = 1300$, 500 mg.) was dissolved in light petroleum (75 ml., B.P. 60–80°) and run into an adsorption tube filled with calcium hydroxide[1] (column of adsorbent 51 × 4 cm.). The chromatogram was developed by washing with several litres of petroleum (B.P. 60–80°). A faint yellow-brown zone formed almost at once, and at the end of the experiment it had moved nearly half-way down the tube and consisted of two main bands of dark chocolate-brown colour, and, in addition, a complicated series of narrow bands. The column was dried in a stream of nitrogen and pushed out of the tube, transverse sections being cut off and eluted with light petroleum containing methyl alcohol. The residue from each fraction was dried *in vacuo* and weighed before being examined spectroscopically. Table I summarises the results obtained.

This typical adsorption shows that a separation into five main fractions has been accomplished:

(*a*) Faintly coloured thick oil, adsorbed strongly at the top of the tower and relatively weak in vitamin A.

(*b*) Less strongly adsorbed material very rich in the vitamin.

(*c*) Deeply coloured oil from the region including the bands. This, whilst still containing vitamin A, also contains a red substance.

(*d*) The material immediately below the colour, with which it overlaps.

(*e*) The least strongly adsorbed material, small in amount and definitely yellow in colour. This is markedly different from all the other fractions.

[1] The preparation of a satisfactory column of calcium hydroxide is much more difficult than that of one of alumina, and since the success of this experiment depends entirely on the use of an evenly packed column the following directions are given. The calcium hydroxide is vigorously shaken with several volumes of petroleum and the mixture allowed to stand for half an hour with occasional gentle agitation to remove air bubbles. The tube is filled with this mixture in several small portions, each portion being carefully stirred and allowed to settle before adding the next. The preparation of a satisfactory column takes several hours.

Table I.

Adsorption of vitamin A on $Ca(OH)_2$ (T_{29}). Starting material, 500 mg. of concentrate $E^{1\%}_{1\,cm.}$ 617 $m\mu$ = 3680, 583 $m\mu$ = 1800, 328 $m\mu$ = 1300.

Fraction	Length of zone (cm.)	Colour of zone	Colour of extract	Wt. of extract (mg.)	Reaction with $SbCl_3$: Colour	Absorption bands ($m\mu$)	$E^{1\%}_{1\,cm.}$ 617 $m\mu$	583 $m\mu$	660 $m\mu$	+7-methyl-indole (max. in $m\mu$)	Ratio of intensities 617:583 $m\mu$	Ultra-violet absorption	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	280 $m\mu$
1	3	White	Pale yellow	64	Blue	622 (weak 697)	802	470	—	580	1·7 : 1	Broad summation 280+328 $m\mu$ bands	250	—
2	6	White	Yellow	89	Blue	620 (weak 700)	3100	1420	—	580	2·2 : 1	328 $m\mu$ band	1080 (approx.)	—
3	7	Dirty yellow	Red	142	Blue	620	3420	1740	—	583	2 : 1	328 $m\mu$ band	1280	—
4	5	Two chocolate-brown bands	Red	62	Blue	620 and satellite 583	1450	850	—	580 and 655	1·6 : 1	Single 328 band. Inflexion 430 $m\mu$	550	—
5	10	Red-pink	Red	18	Slate-blue	Two clearly 645, 583	360	370	340	580 and 650	1 : 1	328 band. Inflexion 430 $m\mu$ Absorption 280 $m\mu$	375	280
6	12	Dirty yellow	Red	7·3	Violet-blue	One band only, 582 (642 on fading)	270	410	—	582 and 642 on fading	0·7 : 1	328 band. Inflexion 420 $m\mu$ Absorption 280 $m\mu$	317	190
7	8	Pale yellow	Bright yellow	0·8	Blue	617 and 580–585	(1400)	(1080)	—	580	1·3 : 1	328 band. Inflexion 370 $m\mu$	(660)	—
8	Filtrate	Pale yellow	Yellow	24	Blue	620 (579)	606	342	—	579 and 625	1·8 : 1	Triplet of bands at 349, 369 and 390 $m\mu$ + 330 $m\mu$ and 278 $m\mu$ summation	150	—

Table II. *The coloured zones.*

Exp. No.	Adsorbent	Colour	Antimony trichloride test: Maxima ($m\mu$)	$E^{1\%}_{1\,cm.}$ 650 $m\mu$	617 $m\mu$	583 $m\mu$	Ratio of 617/583 $m\mu$ intensities	Bands 7-Methylindole + $SbCl_3$ ($m\mu$)	Ultra-violet absorption: Maxima ($m\mu$)	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	280 $m\mu$
T_{18}	Al_2O_3	Blue	620 and 583	—	1070	890	1·2 : 1	580	330 and (410) broad	870	—
T_{21}	$Ca(OH)_2$	Blue	660, 617 and 578	Three bands equal in intensity			1 : 1	660 and 583	—	—	—
$T_{29\,(5)}$	$Ca(OH)_2$	Slate-blue	645 and 583	340	360	370	1 : 1	650 and 583	328 (430)	375	280
C_{12}	Al_2O_3	Blue	643 and 583	240	240	250	1 : 1	645 and 585	Broad and indefinite	206	216
C_{14}	Al_2O_3	Slate-blue	655, 620 and 585	84	122	146	0·8 : 1	656 and 585	Indefinite	126	220
C_{15}	Al_2O_3	Blue	617 and 585	76	218	200	1·1 : 1	660 and 582	—	—	—

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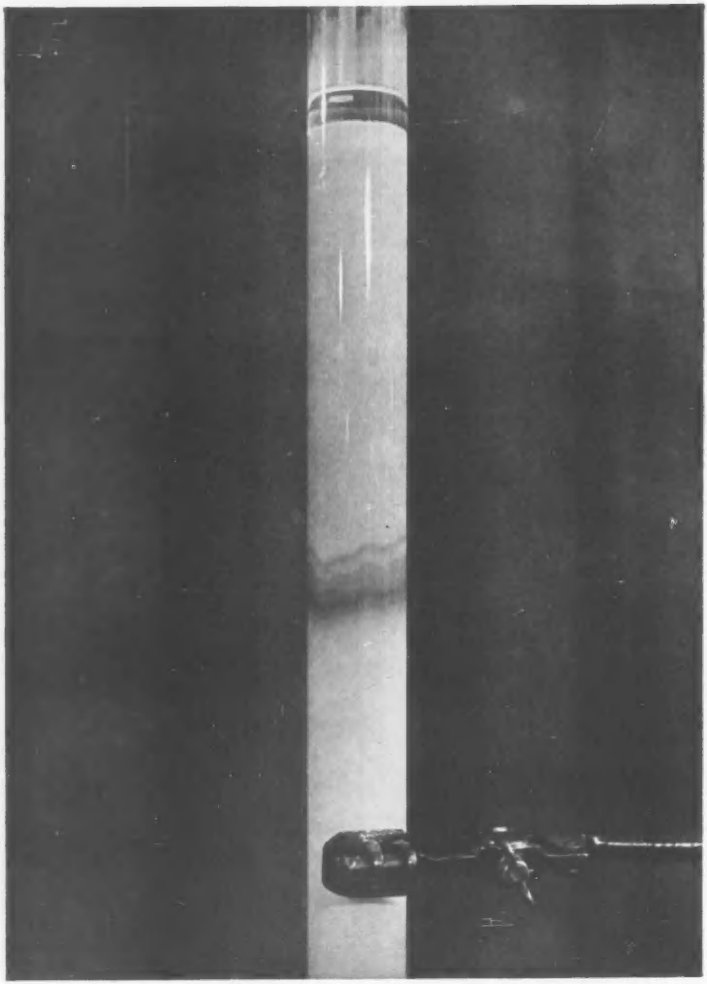

Tube of $Ca(OH)_2$ showing coloured zones produced when a vitamin A concentrate is adsorbed from light petroleum solution.

It is important to note that although 500 mg. of concentrate were originally used in this experiment, only 417 mg. were recovered, and further that, taking the usual criteria for vitamin A, the total amount of this substance recovered was as follows:

By the 328 $m\mu$ band the vitamin recovered $= 52$ %
„ 617 $m\mu$ „ „ } $SbCl_3$ test $= 50{\cdot}4$ % } mean 52 %.
„ 583 $m\mu$ „ „ } „ $= 53{\cdot}8$ %

Thus in this experiment some 18 % of the material was lost mechanically, being adsorbed so strongly that it could not be wholly eluted even by boiling methyl alcohol, whilst a further 30 % was converted into material giving no reactions characteristic of the vitamin.

The coloured zones.

To isolate the coloured fraction either aluminium oxide[1] or calcium hydroxide[2] can be used. With the former the solute is very strongly adsorbed and the colour is produced as a compact dark brown band. As obtained from this adsorbent, however, the coloured fraction contains much vitamin A even after several re-adsorptions. With calcium hydroxide the adsorption is much weaker, the colour in the tube being much more pale and diffused, but the separation of the vitamin from the colour is decidedly more effective even in one adsorption. The relative adsorbing powers of the two solids will be seen from the following data. A tube of dimensions 40×5 cm. when filled with alumina will adsorb the whole of 20 g. of vitamin concentrate, whereas the same tube filled with calcium hydroxide will only hold about 0·5 g. of the same concentrate after washing with light petroleum.

When the coloured band is eluted with methyl alcohol-petroleum and the mixture washed with water to remove alcohol, it is found that the solution so obtained has a much more intense red colour than that originally used. Thus, in one case the original solution was matched by a standard solution of azobenzene. After adsorption the recovered material had to be diluted 12 times to match the standard colour. It is therefore clear that colour is produced during the adsorption process, probably by the catalytic action of the adsorbent on some constituent of the concentrate. This production of colour cannot be reproduced by merely shaking a solution of the material with adsorbent; apparently it is only to be effected by the slow washing of the vitamin concentrate over the adsorbent, such as occurs in a column. During the actual adsorption, colour is produced continuously and washed down into the coloured zone. On passing a solution of the material extracted from that part of the column immediately above the coloured zone (that is the region richest in vitamin A) through another column and developing the chromatogram as usual, a broad red band is again produced, and the process may be repeated several times with the continuous production of colour. It thus seems probable that vitamin A itself is the precursor of the colour, which possibly accounts for the 30 % destruction of the vitamin during adsorption.

The colour is obviously complex in nature, appearing in a well-developed chromatogram as a number of narrow red bands (at least 5) superimposed one on the other (see Plate XI). It is usually obtained as a dark red oil which always contains some vitamin A and from which no solid derivative has yet been obtained. It slowly fades in the dark with the production of a white decomposition product insoluble in petroleum, and is not acted upon by boiling caustic alkali or by carbonyl reagents.

[1] Brockmann's Al_2O_3 for adsorption analysis. E. Merck, Darmstadt.

[2] Calcium hydricum pulvis alcoholisatus, from A. G. vorm B. Siegfied, Zofingen, Switzerland.

The combustion results agree approximately with $C_{20}H_{30}O$ required for vitamin A, but the mol. wt. is high, suggesting the presence of polymerised material. (Found: C, 82·5; H, 10·7 %. Mol. wt. 444, 434. $C_{20}H_{30}O$ requires C, 83·9; H, 10·5 %. Mol. wt. 286.)

The *absorption spectra* of the colour concentrates always show the presence of the 328 $m\mu$ band associated with vitamin A. In addition there is absorption in the visible region in the form of a broad inflexion on the vitamin A curve near 400–430 $m\mu$ (Fig. 1). No evidence of fine structure in the absorption band has been obtained such as one observes in the case of carotene and many carotenoids.

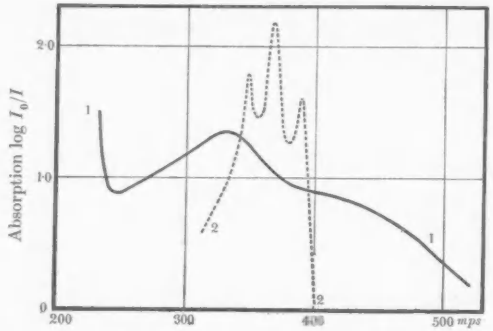

Fig. 1. Absorption spectra of (1) the extract from the coloured zone, 0·0036 % in alcohol, 1 cm. cell; (2) the extract from the bright yellow zone least easily adsorbed, 0·004 % in alcohol, 1 cm. cell [T_{36} (ω)].

With antimony trichloride the coloured fractions give a blue solution showing the absorption spectrum characteristic of vitamin A but, in addition, increased absorption near 583 $m\mu$ and a new band at 642–660 $m\mu$, the exact location of which is probably dependent on the other absorption present. This band is almost certainly due to the red substance and, like the 583 $m\mu$ band in the vitamin A blue colour, is not suppressed by 7-methylindole. Thus in some fractions (*e.g.* C_{14} and T_{21}, Table II) the blue antimony trichloride solution shows three bands near 660, 617 and 580 $m\mu$. When 7-methylindole is added before the antimony trichloride, the 617 $m\mu$ band is no longer visible, only the 660 and 580 $m\mu$ bands remaining.

The conclusions to be drawn about the nature of the colour are that it is due to a substance difficult to characterise, almost certainly derived from vitamin A, and very probably, as obtained by us, a complex mixture of polymerised material containing some unchanged vitamin.

The fractions immediately above the colour.

The zone immediately above the colour was found to be richest in vitamin A. The fractions were syrupy and usually of an orange-yellow colour. In alcoholic solution they exhibit a clear absorption band of high persistence at 328 $m\mu$ and correspond to the β-vitamin A of Karrer, Walker *et al.* [1933]. These workers claim that this material differs from ordinary vitamin A in that it shows only one absorption band at 622 $m\mu$ in the blue antimony trichloride solution. In all the fractions examined by us the only band visible in the spectroscope lies at 617–622 $m\mu$, but if intensity measurements are made at 617 and 583 $m\mu$ the

ratios are found to be approximately 2 : 1, as has always been observed with the purest vitamin A preparations. Moreover, if a small quantity of 7-methylindole is added to the preparation before the antimony trichloride, only a clear band at 583 $m\mu$ is seen, at nearly half the intensity of that originally present at 617 $m\mu$. By adding still less 7-methylindole the 617 $m\mu$ band is only partially suppressed and can be observed side by side with the 583 $m\mu$ band, thus proving that the inhibitor does not merely displace the 617 $m\mu$ band but, by reducing its intensity, reveals the presence of the previously masked 583 $m\mu$ band.

Table III shows some collected data on a number of these fractions, and it is clear that in all cases the ratio of the intensities of absorption at 617 and 583 $m\mu$ is approximately 2 : 1.

Table III. *Fractions immediately above the colour (rich in vitamin A).*

Exp. No.	Adsorbent	Antimony trichloride $E^{1\%}_{1\,cm.}$ 617 $m\mu$	Antimony trichloride $E^{1\%}_{1\,cm.}$ 583 $m\mu$	Ratio of intensities 617 : 583 $m\mu$
T_{36}	Al_2O_3	3930	1900	2·07
C_{25}	Fuller's earth + $Ca(OH)_2$	3430	1730	1·98
T_{34}	$Ca(OH)_2$	3100	1560	1·98
T_{33}	$Ca(OH)_2$	3970	1850	2·14
T_{32}	$Ca(OH)_2$	3860	1760	2·19
T_{31}	$Ca(OH)_2$	3540	1700	2·08
T_{29}	$Ca(OH)_2$	3420	1740	1·97
T_{28}	$Ca(OH)_2$	3310	1700	1·94
T_{27}	$Ca(OH)_2$	3940	1970	2·0
T_{19}	Al_2O_3	3630	1850	1·96
T_{14}	$Ca(OH)_2$	4720	2350	2·0
T_9	$Ca(OH)_2$	3460	1905	1·82
T_5	Al_2O_3	3510	1740	2·01
			Average	2·01

Thus, Karrer's β-vitamin A is qualitatively identical with what has been previously prepared by adsorption and by distillation. With regard to the above author's claim to have prepared a concentrate having $E^{1\%}_{1\,cm.}$ 328 $m\mu = 1700$, we have also obtained values approaching this by adsorption, but products of this high potency are rare, lower values being more usual. In this respect the adsorption method is very little superior to the distillation method where values of $E^{1\%}_{1\,cm.}$ 328 $m\mu = 1600$ have been obtained.

Vitamin A thus gives with antimony trichloride a blue colour exhibiting two absorption bands, one only of which is readily suppressed by the addition of certain reagents (*e.g.* 7-methylindole). The two bands must be derived from two different chromophoric centres in the same molecule, only one of which is affected by 7-methylindole. That this state of affairs is possible is clearly shown by the following facts. We have observed that pure lutein and pure zeaxanthin give a blue colour with antimony trichloride, exhibiting an absorption band at 617 $m\mu$ and a weaker satellite band near 585 $m\mu$ (unpublished work). These bands are very similar to those due to vitamin A, except that their intensities are only about one-tenth of those characteristic of the vitamin. If a trace of 7-methylindole be added to these pure substances before the antimony trichloride reagent, the resulting colour shows only one band at 585 $m\mu$, the band at 617 $m\mu$ having been entirely suppressed.

The top zones of the adsorption tubes.

The results of the examination of the material from the top zones of the adsorption columns are given in Table IV. Absorption spectrum measurements indicate the presence of some vitamin A, together with other material absorbing selectively at 270–280 $m\mu$ and giving a violet colour with antimony trichloride possessing an absorption band near 580 $m\mu$. This material thus agrees in its properties with the strongly adsorbed "580 chromogen" of Eekelen *et al.* [1931; 1932] who have reported the separation of "two chromogens" from vitamin A by means of fuller's earth. The adsorbed fraction on recovery was found to give a violet colour with antimony trichloride, showing only one band near 580 $m\mu$, whilst the unadsorbed fraction gave a colour showing only one obvious maximum at 620 $m\mu$. The fact that the "580 chromogen" is so strongly adsorbed suggests that it consists of partially oxidised vitamin A. We have submitted this hypothesis to direct test by carefully oxidising vitamin A with very dilute chromic acid, using the method of Kuhn and Brockmann [1933], and have actually obtained a product showing selective absorption in the ultraviolet at 275–280 $m\mu$ and giving a violet colour with antimony trichloride with an absorption maximum at 572–580 $m\mu$ (*cf.* Table IV). Products of this nature have also been frequently obtained by other oxidative treatments of vitamin A [Morton and Heilbron, 1928; Morton *et al.*, 1931; Edisbury *et al.*, 1932; Brockmann and Tecklenburg, 1933].

The fractions immediately below the colour.

It has been shown that the red substance is actually formed in the tube during adsorption and is therefore diffused somewhat into the fractions immediately above and below it. This lower fraction appears to correspond to the "α-vitamin A" (hepaxanthin) of Karrer, Walker *et al.* [1933] and Karrer [1934]. These authors claim to have obtained by adsorption of rich vitamin A concentrates on calcium hydroxide, in addition to the main fraction (β-vitamin A) adsorbed in the upper part of the tube, a small amount of an isomeric substance (α-vitamin A) in an impure state from the lower zones. α-Vitamin A is characterised by an absorption band at 270–280 $m\mu$ and by a violet antimony trichloride colour showing only one band at 580 $m\mu$, which, however, soon disappears giving place to one at 620 $m\mu$. It is further suggested that the latter develops at the expense of the former, presumably owing to the passage of the labile "α-vitamin A" into the "β-form."

Our detailed examination of the material from this zone shows that, in alcoholic solutions, it exhibits an absorption band at 275–280 $m\mu$ accompanied in most cases by the usual 328 $m\mu$ band (*cf.* Fig. 1). With antimony trichloride it gives a violet or blue colour showing a band near 580 $m\mu$ (cf. Table V). These observations are in accord with those of Karrer and his collaborators and further, we confirm the observation that the violet antimony trichloride solution rapidly changes on standing, and that a band near 620 $m\mu$ (or 640 $m\mu$, if colour was present in the fraction) appears to increase in intensity at the expense of the 580 $m\mu$ band. Careful and repeated spectrophotometric measurements of the intensities of the two bands during this fading process, however, reveal that the 620–640 $m\mu$ band is actually present all the time but is initially masked by the strong 580 $m\mu$ band. This latter fades so rapidly, however, that the band at 620–640 $m\mu$ becomes more and more visible in the spectroscope, although in no case have we observed it to increase in intensity as the intensity of the other band decreases.

Table IV. *Fractions from the tops of the adsorption columns.*

Antimony trichloride test

Table IV. *Fractions from the tops of the adsorption columns.*

Exp. No.	Adsorbent	Wt. of fraction mg.	Antimony trichloride test: Colour	Maxima in mμ	$E^{1\%}_{1\,cm.}$ 617 mμ	583 mμ	573 mμ	Ratio of 617 : 583 mμ intensities	7-Methylindole $SbCl_3$. Maximum in mμ	Ultraviolet absorption: Maxima in mμ	$E^{1\%}_{1\,cm.}$ 328 mμ	280 mμ
C_7	Al_2O_3+ "Fasertonerde"	88	Purple-blue	617 (583)	1380	1160	—	1·2 : 1	578	—	—	—
T_{17}	$Ca(OH)_2$	53	Blue	617	945	580	—	1·6 : 1	—	—	—	—
T_{29}	$Ca(OH)_2$	64	Blue	622 (697)	802	470	—	1·7 : 1	580	Indefinite 328 and 280	250	—
C_{23}	Fuller's earth	30	Violet-blue	620, 575	283	—	224	1·3 : 1	577	—	—	—
C_{25}	Fuller's earth	79	Violet-blue	617, 582	1030	1030	—	1 : 1	582	270 inflexion at 328	670	950
C_{27}	Fuller's earth	7·5	Violet-blue	617, 583	475	485	—	1 : 1	584	328 and 280 summation	—	342
C_{29}	Fuller's earth	13	Violet-blue	588	870	1440	—	0·6 : 1	588	330 and 280 summation	1000	900
Oxidation products of vitamin A												
T_{23}	—	42	Violet-blue	618, 578	860	610	—	1·4 : 1	—	328 mμ band and inflexion near 280	334	—
T_{26}	—	60	Strong violet	617, 573	833	—	1040	1 : 1	573	328 mμ band and absorption 260–300	260	230

Table V. *The zones immediately below the colour.*

Exp. No.	Wt. of fraction mg.	Adsorbent	Antimony trichloride test: Colour	Maxima in mμ	$E^{1\%}_{1\,cm.}$ 617 mμ	583 mμ	Ratio of 617 : 583 mμ intensities	7-Methylindole + $SbCl_3$ bands	Ultraviolet absorption: Maxima in mμ	$E^{1\%}_{1\,cm.}$ 328 mμ	280 mμ
T_{34} (4)	12·6	$Ca(OH)_2$	Violet-blue	628 and 582	178	178	1 : 1	642 and 583	—	—	—
T_{22}	17·6	$Ca(OH)_2$	Violet	622 and 577	90	105	0·85 : 1	623 and 577	330 inflexion band near 280	54	70
T_{29} (6)	7·3	$Ca(OH)_2$	Violet	582 only	270	410	0·7 : 1	640 and 582	330 band and inflexion 275 mμ	320	210
C_{25}	5	$Ca(OH)_2$ with fuller's earth above	Violet	623 and 580	270	350	0·8 : 1	583	328 inflexion, 275 band	250	465
C_{26}	15	$Ca(OH)_2$ with fuller's earth above	Violet	585	54	72	0·8 : 1	—	275	—	90

Table VI. *Material from the yellow zone (least easily adsorbed).*

Exp. No.	Wt. of fraction mg.	Adsorbent	Location in column	Bands with $SbCl_3$ mμ	$E^{1\%}_{1\,cm.}$ 620 mμ	583 mμ	7-Methylindole + $SbCl_3$ bands	Ultraviolet absorption: Maxima in mμ	$E^{1\%}_{1\,cm.}$ 370 mμ
T_{21}	—	$Ca(OH)_2$	Below colour	622, 578	—	—	622, 577	—	—
T_{28}	30·8	$Ca(OH)_2$	Below colour	622, 578	414	292	625, 579	Triplet of narrow bands max. 366	—
T_{29} (8)	24	$Ca(OH)_2$	Well below colour	620, 579	605	342	625, 579	Triplet max. 370	195
T_{26} (4)	108	Al_2O_3	Just below colour	622	1300	580	624, 580	Triplet max. 349, 369, 390	670
T_{26} (5)	97·3	Al_2O_3	Just below colour	623, 583	1230	554	623, 582	Triplet max. at 348, 370, 387	560
T_{34} (5)*	10·5	$Ca(OH)_2$	Below colour	628, 582	208	180	640, 583	328. Inflexion 390	107

* Contains some of the red colour also.

We do not consider that the optical properties provide evidence that this substance is an isomeric form of the vitamin. Its properties agree with those of the oxidation products of vitamin A found in the top zones and with those of the "580 chromogen" of Eekelen *et al.* It is, however, much less strongly adsorbed, being still obtained below the colour when a solution from which the "580 chromogen" has been removed by preliminary adsorption on fuller's earth is passed through a column of calcium hydroxide (*cf.* Table V). It is thus probable that it is in a lower state of oxidation than the material in the top zones. Euler *et al.* [1934] suggest that the growth-promoting activity of this fraction may be simply explained by the occurrence in it of 10 % of vitamin A itself.

The yellow zone least easily adsorbed.

Using alumina as adsorbent and employing 5–10 g. of vitamin A concentrate a distinct bright yellow band develops well below the other fractions in the adsorption-tube. With calcium hydroxide as adsorbent the yellow band is much more diffuse. On elution a small quantity (2–5 % of original concentrate) of a thick yellow oil is obtained (*cf.* Table VI) differing altogether in properties from the other fractions. Its absorption spectrum (in alcohol) shows sharply defined maxima at 348, 369 and 389 $m\mu$ (Fig. 1) and thus resembles the spectra of the typical carotenoids with the maxima displaced from the visible into the near ultraviolet. The antimony trichloride solution has a bluish colour showing absorption maxima at 624 and 579 $m\mu$. On partition of the pigment between 90 % methyl alcohol and light petroleum it goes largely, but not completely, into the alcohol. Attempts to characterise it further have so far been unsuccessful, as on standing in solution the intensity of absorption and chromogenic power rapidly decrease, but we are actively engaged in further work with a view to its more precise characterisation.

SUMMARY.

Complete chromatographic analysis of rich vitamin A concentrates controlled by spectroscopic examination of the alcoholic solutions and of the antimony trichloride blue colours shows that five main fractions may be obtained. In order of adsorption on alumina or calcium hydroxide they are:

(*a*) Strongly adsorbed material which with antimony trichloride gives a violet or violet-blue colour. This corresponds to the similar material obtained by Eekelen *et al.* [1931; 1932] and is considered to consist largely of oxidation products of the vitamin.

(*b*) The main fraction consisting of highly concentrated vitamin A characterised by an absorption band at 328 $m\mu$. This corresponds with Karrer's so-called β-vitamin A, but it is found that, in addition to the band at 620 $m\mu$ in the antimony trichloride blue colour, the usual band at 583 $m\mu$ is also present as a masked maximum. The intensities of absorption at these two wavelengths are approximately 2 : 1, as has always been observed with the purest vitamin A concentrates. It is therefore concluded that the "β-vitamin A" of Karrer is essentially the same substance as the best vitamin A preparations previously obtained.

(*c*) A small amount of strongly coloured red material which appears to be produced from the vitamin during the adsorption process. Its absorption spectrum shows, together with the usual 328 $m\mu$ band of vitamin A, a smooth inflexion near 430 $m\mu$ which is the complement of the red colour. With antimony trichloride it gives the blue colour usually characteristic of vitamin A but

showing an additional absorption band between 640 and 660 $m\mu$. Molecular weight determinations suggest the presence of polymerised material in this fraction.

(*d*) The so-called α-vitamin A (hepaxanthin) of Karrer *et al.* This is only small in amount and shows an absorption maximum at 270–280 $m\mu$ and a violet colour with antimony trichloride exhibiting a 580 $m\mu$ absorption band. In general, this material closely resembles fraction (*a*) in its properties and is therefore probably a decomposition product of the vitamin, but in a lower state of oxidation than the more strongly adsorbed fraction. There is no evidence that this fraction contains an isomeric form of the vitamin.

(*e*) A hitherto unrecorded yellow substance characterised by three distinct absorption bands at 348, 369 and 389 $m\mu$. It gives a blue colour with antimony trichloride showing absorption maxima at 624 and 579 $m\mu$.

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CCXXVIII. A SURVEY OF ANTHOCYANINS. IV.

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The scope and methods of this investigation have been sufficiently explained in earlier Parts of the series [1931; 1932; 1933] and only a few comments are needed here to supplement the results summarised in the sequel.

The progress of parallel synthetic investigations has now enabled the delphinidin and petunidin glucosides to be characterised; the result goes to show that the majority of delphinidin diglycosides may with confidence be classed as delphinidin 3 : 5-dimonosides.

A problem arises in connection with the so-called complex anthocyanidins. These substances are often obtained by hydrolysis of anthocyanins in crude plant extracts, and they are undoubtedly acylanthocyanidins because they yield the anthocyanidins by hydrolysis with alkalis followed by reconstitution with acids. A characteristic example is derived from the dahlia "George Ireland." Its behaviour to our "delphinidin reagent" is that of delphinidin itself, but unlike this anthocyanidin it is largely taken up in the "cyanidin reagent." As the "oxidation test" also indicated delphinidin we mistook this substance for that anthocyanidin in our first examination of it. The difficulty is that the anthocyanin of the dahlia is cyanin, and it is hard to find a position for the acyl group which will fit in with the facts. Possibly the acyl group is introduced in the course of the hydrolysis itself by a migration from sugar hydroxyl to phenolic hydroxyl.

In diagnosing petunidin there are two dubieties: in the first place, admixture of cyanidin or delphinidin is difficult to detect, and in the second place, petunidin is hard to distinguish from a mixture of delphinidin and cyanidin. As far as possible these doubts have been resolved by fractionation of the pigment, but it is not always feasible to do this and "petunidin" should be taken to imply the possibility of delphinidin-cyanidin mixture, especially when it is not the only anthocyanidin present. We have noted a number of cases in which petunidin is present without admixture and no doubt arises in those examples.

We would point out that in Part I no distinction was made between pentose glycosides and monosides; as far as possible the doubtful cases are being reviewed. Pentose-glycoside is used as a generic term to include both aldopentose glucosides (for example, primeverosides) and rhamnoglucosides.

The effect of co-pigments on the distribution numbers of anthocyanins may be very large (compare *Primula sinensis*) and we have included a brief account of some experiments made in collaboration with Mr E. A. H. Roberts, showing a connection between the colour-change and effect on distribution numbers brought about by the addition of papaverine hydrochloride to solutions of chrysanthemin chloride and oenin chloride. Narcotine is a very weak co-pigment for these anthocyanins and correspondingly its effect on the distribution number is slight.

We imagine that the co-pigments combine with the anthocyanins in solution and in so doing break up the associated complexes. Natural co-pigments, more readily soluble in water than in amyl alcohol, behave similarly and greatly reduce the distribution number.

Summary of results.

Abrus precatorius. The scarlet seed-coatings contain an interesting anthocyanin, the investigation of which is hindered by the presence of a large proportion of other substances soluble in amyl alcohol and in water. The coatings were separated and extracted with 1 % methyl alcoholic hydrogen chloride, and the anthocyanin was precipitated from the solution by means of ether. This process was twice repeated using ethyl alcoholic hydrogen chloride and the pigment could then be analysed. It contains two anthocyanins, one of them diglucosidic and the other most probably complex (that is, acylated) diglucoside. According to our tests the anthocyanidin is delphinidin but this is always mixed with another aglucone, possibly cyanidin, but more probably acylated delphinidin. The anthocyanin colour-reactions (violet in sodium carbonate and unusual stability towards sodium hydroxide) do not tally with those of known delphinidin derivatives. It is undoubtedly worthy of study in substance and a preliminary investigation has been made by N. Ghatak (*Bull. Acad. Sci.*, U. P. Allahabad) who regards the anthocyanin (found: C, 50·1; H, 5·4 %) as monoglycosidic and cites the distribution number, 11·1, 10·4. From the description it is evident that the pigment was not isolated in a pure condition.

Abutilon insignia. Brownish crimson flowers, richly pigmented; cyanidin 3-pentoseglycoside.

Agaricus. The pink parts of young mushrooms contain leuco-compounds yielding a water-soluble pigment recalling betanin in its colour reactions.

Allium giganteum. Violet flowers; cyanidin 3 : 5-dimonoside.

Ampelopsis hederacea. The outer parts of the stems contain leuco-anthocyanin → cyanidin. The blue-black berries contain a complex anthocyanin based on cyanidin, together with a little malvidin.

Anemone fulgens. Bright bluish red flowers; petals, pelargonidin 3-glucoside (distribution number of purified anthocyanin, 33, agreeing with callistephin); anthers complex delphinidin diglycoside.

Aquilegia hybrida. The purple leaves contain cyanidin 3 : 5-dimonoside. *A. alpina,* blue-violet flowers; delphinidin 3 : 5-diglycoside. *A. canadensis,* brownish pink flowers; pelargonidin 3-monoside. *A. sibirica,* violet flowers; complex delphinidin diglycoside.

Asparagus. The purplish tips contain a cyanidin diglycoside.

Aubrietia deltoidea. A series of flowers ranging from blue-violet to red-violet ("Church Knowle," "Dr Mules," "Aubrey Prichard," "Excelsa," "Fire King," "Gloriosa") all contained cyanidin 3 : 5-dimonoside. The colour variations must be due to co-pigments as the solution from "Dr Mules" becomes blue and that from, say, "Gloriosa," becomes violet on the addition of sodium acetate (compare Part I).

Beeswax. The presence of leuco-anthocyanin → cyanidin was detected.

Begonia. "Prince Eugene," bluish pink flowers; cyanidin 3-bioside.

Bignonia speciosa. Violet flowers; based on cyanidin and probably complex diglycoside.

Boronia elatior. Rosy carmine flowers; malvidin 3 : 5-diglycoside with a trace of Fe^{+} anthocyanin.

Bougainvillaea. A variety with scarlet bracts collected in Madeira was found to contain a betanin-like anthocyanin. This gives solutions that are much less blue-red than those from other *Bougainvillaea* or from *Beta* and affords an indication of the existence of more than one betanin type.

Browallia demissa. Blue flowers; complex diglycoside of delphinidin, highly co-pigmented.

Brunfelsia calycina var. *macrantha.* Violet flowers; mixture of malvidin 3 : 5-dimonoside and complex dimonoside.

Buddleia colvilei (Himalaya). Rosy magenta flowers; cyanidin pentoseglycoside or complex glycoside.

Burchellia capensis. Orange flowers; cyanidin 3-pentoseglycoside.

Butea frondosa. The gum contained leuco-anthocyanin → cyanidin.

Cactus. Heliocereus speciosus. Crimson; betanin-type anthocyanin.

Calceolaria. "Albert Kent" hybrid, brown centres; cyanidin 3-bioside; brownish violet flowers, petunidin 3-pentoseglycoside was indicated by the tests. The anthocyanin was proved to be not complex.

Calendula tagetes. Reddish brown petals; cyanidin 3-pentoseglycoside.

Callistemon citrinus var. *splendens.* Crimson stamens; co-pigmented cyanidin 3 : 5-dimonoside.

Camellia japonica. Bright bluish red flowers; very clean cyanidin 3-monoside. The solution gives the normal reactions without any treatment.

Canna indica. The scarlet flowers and the leaves contain cyanidin 3-pentoseglycoside and much anthoxanthin, especially in the leaves.

Catechu (pale). A little leuco-anthocyanin → cyanidin.

Cattleya Skinneri. Reddish violet flowers; co-pigmented cyanidin diglycoside, apparently Fe^{--} in the anthocyanin condition (compare *Senecio*).

Celosia cristata. Bluish red flowers contain a betanin-type pigment (also noted by R. Willstätter, private communication); yellow flowers contain a water-soluble, yellow pigment not extracted by ethyl acetate but soluble to some extent in amyl alcohol; scarlet flowers contain a mixture of these colouring matters.

Centaurea montana. Blue flowers with purple centre; a mixture of cyanidin 3 : 5-dimonoside [Willstätter and Everest, 1913] and complex diglycoside of cyanidin.

Cercis siliquastrum. Pink petals; malvidin 3 : 5-dimonoside; the sepals contain a complex anthocyanin based on malvidin.

Cestrum purpureum elegans. Brownish red flowers; mixture of pelargonidin and cyanidin 3-pentoseglycosides.

C. Newellii. Rosy red flowers; the same mixture.

Cinchona rubraceae. The powdered bark contains leuco-anthocyanin → cyanidin.

Cinnamonum. The drug contains leuco-anthocyanin → cyanidin.

Clematis Durandii. The most blue-flowered variety examined; delphinidin diglycoside. *C. Kernesina*, the reddest kind tested; cyanidin diglycoside of unusual type. In both cases a part of the anthocyanin was difficult to hydrolyse. *C. Hendersonii* and *C. Viticella*, reddish violet and bluish violet flowers respectively; delphinidin 3-bioside and some cyanidin derivative in the former. The bluer kinds had more anthoxanthin co-pigment. The colour reactions of the anthocyanins in aqueous alkalis are violet and this in conjunction with relative stability to sodium hydroxide indicates the 3-substituted delphinidin type.

Clianthus Dampieri. Crimson flowers; chiefly pelargonidin 3-monoside with cyanidin 3-monoside as secondary anthocyanin.

Clivia nobilis. Pinkish orange flowers; pelargonidin 3-pentoseglycoside. *C. miniata*, orange-scarlet flowers; pelargonidin 3-saccharide, probably 3-bioside.

Cocos nucifera. The mesocarp contains leuco-anthocyanin → cyanidin.

Coleus shirensi. Violet flowers; complex delphinidin 3 : 5-dimonoside.

Colombia thyracanthus rutilans. Dull vermilion flowers, pelargonidin 3-rhamnoglycoside (the distribution tallied with that of the 3-rhamnoglycoside of the scarlet gloxinia).

Crambe maritima. The purplish tips contain a diglycoside anthocyanin very difficult to hydrolyse by hydrochloric acid in the usual way. After boiling with aqueous sodium hydroxide it was readily hydrolysed by hydrochloric acid, yielding cyanidin.

Crassula coccinea. Bluish red flowers, co-pigmented cyanidin 3-monoside.

Cyclamen. Karrer and Widmer [1926] found a malvidin 3-glucoside in *C. persicum* Mill. Other pigments are, however, present in some of the varieties. A mauve-red-flowered kind contained pure malvidin 3-monoside and very little anthoxanthin. Sutton's "Giant Crimson" is also of this type. Sutton's "Firefly," scarlet flowers, contained peonidin 3-pentoseglycoside. "Giant Salmon Scarlet" and "Silver-leaf Salmon" are of the same type. The acid and alkaline solutions are a little bluer than those of peonidin 3-glucoside (synthetic), but this is due to co-pigment and if tannin were added to the synthetic anthocyanin solution, a good match resulted. "Giant Mauve" contains malvidin 3 : 5-dimonoside.

"Hydrangea Pink" contains much anthoxanthin, and the results in regard to the anthocyanin were not clear; the anthocyanidin is malvidin. "Vulcan," deep red flowers; malvidin 3-monoside

which was directly compared, after purification, with oenin and found to have an identical distribution number and other properties.

Dahlia. The variety "George Ireland," mauve flowers, was stated in Part II [1932] to contain a delphinidin diglycoside. On hydrolysis the anthocyanidin has indeed some of the properties of delphinidin, but it is really a complex cyanidin. Cyanidin, showing the correct distribution and other properties, can be obtained only after a preliminary hydrolysis with sodium hydroxide.

Delphinium. "Los Angeles," pink flowers; pelargonidin 3-bioside of the type unstable to sodium hydroxide.

Dipterocarpus Zeylanicus. The bark contains leuco-anthocyanin → delphinidin and less cyanidin.

Echinops bannaticus. Lavender-blue flowers; delphinidin 3 : 5-dimonoside possibly accompanied by a cyanidin derivative.

Epacris miniata superba. Bluish red flowers; cyanidin 3-monoside.

Erica curviflora var. *Burchellii.* Cyanidin 3-pentoseglycoside.

Eriobotrya japonica. The testa contains leuco-anthocyanin → cyanidin.

Euonymus japonicus. The bluish red fleshy aril contains much anthoxanthin and a cyanidin derivative. The colourless testa becomes red in light and air and contains leuco-anthocyanin → cyanidin.

Fagus sylvatica. The green leaves that have been protected from light contain leuco-anthocyanin → cyanidin.

Gentiana campestris. Reddish violet flowers and stems; delphinidin 3-pentoseglycoside.

Geranium phaeum. Maroon flowers; the anthocyanin has a pentoseglycosidic type of distribution, and it is based on delphinidin and its methyl ethers. The colour reactions suggest a 3-saccharide.

Gladiolus Colvillei. "Spitfire," salmon flowers tinged blue-red; pelargonidin 3-pentoseglycoside.

Gloxinia speciosa. "Prince Albert," violet flowers; cyanidin 3-pentoseglycoside with some Fe⁻ anthocyanin. "Roi des Rouges" and "Kaiser Friedrich," crimson, contain pelargonidin 3-pentoseglycoside. The latter anthocyanin is rather stable towards aqueous sodium hydroxide.

Grevillea punicea. Vermilion flowers; cyanidin 3-pentoseglycoside.

Haemanthus Katherinae. Salmon-pink flowers; pelargonidin 3-bioside having an abnormally low distribution. As with the anthocyanin of *Clivia miniata*, the action of sodium hydroxide gives relatively slow decoloration to a greenish shade. That of *Papaver orientalis*, also pelargonidin 3-bioside, is immediately changed to yellow. The pelargonidin 3-bioside of the orange-scarlet nasturtium is much more stable. It is evident that there is some unexplained cause of this striking difference in behaviour.

Helipterum Manglesii. Bright pink flowers; cyanidin 3 : 5-dimonoside.

Helleborus. Rose-pink flowers contained leuco-anthocyanin → cyanidin and some colouring matter not soluble in dilute hydrochloric acid.

Hydrangea hortensis. The ordinary red-flowered varieties such as "Parzival" appear to contain complex delphinidin diglycoside early in the season; later on this complex character is not observed. Some faded red flowers were extracted with 1 % hydrochloric acid and the pale-coloured solution discarded; on heating the residue with more 1 % hydrochloric acid, a considerable amount of leuco-anthocyanin → complex cyanidin was obtained. The pigment of blue flowers under similar conditions was much more stable.

Impatiens minimum. Garden hybrid with pinkish violet flowers; complex malvidin 3 : 5-dimonoside. Rosy scarlet flowers; complex peonidin 3 : 5-dimonoside.

Ipomaea Horsfalliae var. *Briggsi.* Bluish red flowers; pelargonidin 3 : 5-dimonoside. *I. tricolor*, sky-blue flowers which become rosy mauve in a few hours and are then acid to litmus; malvidin 3 : 5-dimonoside.

Iris miranda. Bluish violet flowers; complex delphinidin diglycoside. *I. Kharput*, violet; also complex delphinidin diglycoside, but not identical with *I. miranda* unless there is a very large co-pigment effect. The distribution number of *I. Kharput* is the higher, and with sodium acetate it gives a violet coloration, *I. miranda* a blue coloration. "Rose Unique," reddish mauve flowers; complex petunidin diglycoside; the colour reactions closely resemble those of the *Iris Kharput* pigment.

Ixia crateroides. Red flowers; cyanidin 3 : 5-dimonoside with much anthoxanthin.

Kalanchoe flammea. Orange-scarlet flowers; carotenoid along with cyanidin 3 : 5-dimonoside. *K. globulifera coccinea*, scarlet flowers; cyanidin 3-saccharide, very much anthoxanthin obscured distribution ratio observations.

Krammeria. The tincture contains leuco-anthocyanin → pelargonidin or, less probably, peonidin.

Lathyrus odoratus. The study of the genetics of the sweet-peas from the point of view of the pigments of the flowers has been undertaken in collaboration with Miss R. Scott-Moncrieff and her colleagues of the John Innes Horticultural Institution. It is not necessary, therefore, to give the results in detail at this stage but a broad summary is desirable. The most orange-red of the sweet-peas contain pelargonidin 3-monoside, always mixed with more or less pelargonidin 3 : 5-dimonoside. Even the variety "2LO" is now of this type, many specimens having been examined. Next in the series we find varieties with pelargonidin 3 : 5-dimonoside, a good example being "Colorado." This pigment may be associated with much anthoxanthin as in "Ascot." Pelargonidin 3 : 5-dimonoside is also associated with complex pelargonidin 3 : 5-dimonoside, an example being "Firefly." The red sweet-peas contain peonidin 3 : 5-dimonoside as chief anthocyanin accompanied by more or less cyanidin 3 : 5-dimonoside. For example, "Honour" is nearly free from cyanidin derivatives, "Captain Blood" has more and "Miss Hunt" a good proportion. One variety which has been examined contains nearly pure cyanidin 3 : 5-dimonoside. In diagnosing cyanidin 3-bioside in "Captain Blood" and pelargonidin 3-bioside in "2LO" we were probably misled by mixtures simulating pure products.

The violets, maroons and blues include pure malvidin 3 : 5-dimonoside and delphinidin 3 : 5-dimonosides with certainty. The cases diagnosed as petunidin 3 : 5-dimonoside are in all probability correctly so described, although the distinction between petunidin and cyanidin-delphinidin is difficult to establish. A malvidin-delphinidin mixture can, however, be differentiated with ease.

L. undulatus. Reddish violet flowers; malvidin 3 : 5-dimonoside.

Linum grandiflorum var. *rubrum*. Crimson-scarlet flowers; re-examination showed the anthocyanidin to be delphinidin without cyanidin admixture. The p_H of the red flowers of the annual flax is lower than that of blue flowers, and as is also the case with *Hydrangea* and *Delphinium*, the anthocyanin concentration is higher in the flowers with a redder shade; the same thing is true of violas as may be seen on inspection.

Linum perenne. Blue flowers; delphinidin 3 : 5-dimonoside.

Lobelia cardinalis. "Queen Victoria," scarlet flowers; pelargonidin 3-monoside, probably identical with callistephin as the anthocyanin was purified and found to have a distribution number 32·5. In every other respect it tallied with callistephin.

Lupinus polyphyllus. "Northern Fire," blood-red flowers, and "Hades," bluish-red; cyanidin 3-monoside with much anthoxanthin. "Chelsea Blue" and "Plato," blue flowers; much anthoxanthin and delphinidin 3-monoside. "Evening Tints," pale salmon flowers; pelargonidin 3-saccharide having a high distribution, actually more like pentoseglycoside than monoside. "Simpson 316," pink, contains the same anthocyanin. "Sailor Boy," violet flowers, and "Athene," reddish violet; petunidin-delphinidin 3-monoside.

Masdevillia Harryana purpurea. Bluish red flowers; cyanidin diglycoside and carotenoid. The anthocyanin is Fe^{-} and gives a violet coloration with aqueous sodium carbonate; on the addition of sodium hydroxide this does not become blue but is decolorised (a faint blue being more stable); the anthocyanin is of a novel type.

Meconopsis Baileyi. The anthocyanin of the blue flowers is a complex cyanidin diglycoside giving a complex cyanidin on hydrolysis.

Medinilla magnifica var. *superba*. Deep pink flowers; delphinidin diglycoside.

Mesembryanthemum. The pink flowers of the succulent leaved species common near Gibraltar contain a betanin-like anthocyanin.

Michelia fuscata. Purplish brown flowers; cyanidin 3-pentoseglycoside.

Mimulus cardinalis. Scarlet flowers; pelargonidin 3-monoside.

Morus nigra. Mulberries contain pure cyanidin 3-monoside.

Nemesia strumosa Suttoni. Bluish red flowers; cyanidin 3-saccharides, a mixture most closely approximating to the behaviour of a pentoseglycoside.

Nymphaea gigantea. Blue flowers; delphinidin complex diglycoside.

Oenothera agari. Pink petals; cyanidin 3-pentoseglycoside and a little complex anthocyanin.

O. rubricalyx. Red calyx; pure cyanidin 3-pentoseglycoside.

Opuntia. The fruits of the prickly pear contain a betanin-type anthocyanin.

Origanum vulgare. The dried leaves contain leuco-anthocyanin → cyanidin.

Orychophragnus violaceus. Violet-blue flowers; cyanidin 3 : 5-dimonoside.

Oxalis rosea. Reddish mauve flowers; malvidin 3 : 5-dimonoside.

Paeonia officinalis. "Delavaye," maroon flowers contain a mixture of peonidin 3 : 5-dimonoside and cyanidin 3-monoside [*cf.* Willstätter and Nolan, 1915].

Passiflora racemosa. Brownish pink petals; pelargonidin 3-monoside; red-violet stamens, cyanidin 3-monoside.

Pentapterygium serpens. Brownish red flowers; cyanidin 3-monoside.

Pentas carnea. Pale mauve flowers; pigment behaved abnormally. *P. coccinea,* scarlet flowers; cyanidin 3-bioside, with no abnormality.

Petracum erodinum. Deep mauve flowers; malvidin 3 : 5-dimonoside.

Petunia hybrida. "Silver Lilac," violet flowers; malvidin 3 : 5-dimonoside. The anthocyanidin is Fe^{-} and contains no trace of petunidin [Willstätter and Burdick, 1917].

Phaseolus multiflorus. The scarlet runner "Scarlet Emperor" contains pure pelargonidin 3-bioside in its flowers.

Phlox divaricata var. *Laphami.* Violet-blue flowers; delphinidin 3 : 5-dimonoside mixed with a trace of a second anthocyanin.

Pinus excelsa. The young cones obtained from Sweden contain cyanidin 3-monoside and also leuco-anthocyanin → cyanidin.

P. sylvestris and other pines have leuco-anthocyanin → cyanidin in the cones.

Platytheca galioides. Violet-blue flowers; complex malvidin diglycoside. The reactions were much obscured by the presence of substances which caused the precipitation of the anthocyanin in alkaline solution.

Plumbago rosea. Bright pink flowers; complex peonidin 3-saccharide.

Polygala myrtifolia. Rosy magenta flowers; cyanidin 3 : 5-dimonoside.

Polygonum amplexicaule. Dull red flowers; cyanidin pentoseglycoside.

Pontideria. A blue-violet flowered species; delphinidin 3 : 5-dimonoside.

Potentilla argyrophylla var. *astrosanguinea.* Scarlet flowers; cyanidin 3-monoside.

Primula mollis. Red-violet flowers; pure malvidin 3 : 5-dimonoside. *P. malacoides,* "The Prince," violet-rose; malvidin 3 : 5-dimonoside; other varieties such as "The Dawn," pink, and "Dainty," pinkish mauve, and "Princes Mary," mauve, contain malvidin 3 : 5-dimonoside with more anthoxanthin. *P. obconica,* "Giant Crimson"; delphinidin 3 : 5-dimonoside. *P. officinalis,* reddish brown flowers; hirsutidin or, less probably, malvidin pentoseglycoside with much co-pigment. *P. sinensis,* "Reading Ruby," or "Giant Terracotta," contains malvidin 3-monoside, not dimonoside as formerly stated. The very large amount of co-pigment holds the pigment in the aqueous layer so that the distribution appears diglycosidic; it is monoglycosidic after purification. The leaves of *P. sinensis* contain a leuco-anthocyanin.

Prunus persica. The pink petals and sepals contain an anthocyanin yielding a complex cyanidin on hydrolysis and some leuco-anthocyanin → cyanidin. The leaf-curl contains much anthoxanthin and cyanidin 3-monoside. The leaves of *P. pissardii* remain green if not exposed to light (compare Part I) but contain leuco-anthocyanin → cyanidin.

Pterocarpus marsupium. The kino contains leuco-anthocyanin → cyanidin and a little delphinidin.

Pyrus malus Niedzwetzkyana. The fruit develops red stains when injured and the uncoloured parts contain leuco-anthocyanin → cyanidin. We neither suggest nor reject the view that the stains arise from transformation of the leuco-anthocyanin.

Ranunculus asiaticus. Orange flowers; cyanidin 3-bioside with much anthoxanthin and carotenoid; a salmon-pink-flowered kind was the same but free from carotenoid. A scarlet garden hybrid *Ranunculus* contained cyanidin 3-bioside, carotenoid and anthoxanthin; a deep rosy pink hybrid contained cyanidin 3-bioside and anthoxanthin, exactly the same difference as in the *R. asiaticus* varieties.

Rehmannia angulata. Deep bluish pink flowers; cyanidin 3-pentoseglycoside.

Rheum officinale. The drug *Rhei radix* contains leuco-anthocyanin → cyanidin. *R. rhaponticum*, green leaves contain leuco-anthocyanin → cyanidin.

Rhododendron Griersonianum. Vermilion and *R. euchaites*, scarlet, both contained pure cyanidin 3-monoside. This has been previously recorded for cultivated varieties but these are species from N.W. Yunnan.

Richardia Elliottiana. The yellow petals contain leuco-anthocyanin → cyanidin.

Rochea ciliata. Rosy pink flowers; pelargonidin 3-pentoseglycoside and some cyanidin derivative. The reactions were much obscured by tannin.

Rosa. The orange-red polyantha roses such as "Gloria Mundi" and "Prince of Orange" contain pelargonidin 3 : 5-dimonoside in a pure condition. On one plant some of the flowers were pink and some orange; the pink contained cyanin and the orange pelargonidin dimonoside. "Veilchen Blau" appeared to develop less blue-red flowers in this dry season (1934) than normally. The bluer petals were separated and found to contain complex cyanidin 3 : 5-dimonoside with some cyanin. The redder petals contained less of the complex pigment.

Salpiglossis. Dark crimson hybrid; complex diglycoside of petunidin (or cyanidin-delphinidin).

S. sinnata. Brown flowers; complex diglycoside of petunidin together with carotenoid.

Sambucus racemosa. The red berries contain cyanidin 3 : 5-dimonoside.

Scabiosa. "Azure Fairy," lavender-blue flowers; delphinidin diglycoside. "Black Prince," crimson flowers; cyanidin 3-pentoseglycoside. "Fire King," bluish red flowers; pelargonidin 3-pentoseglycoside.

Seaweeds. We have not observed normal anthocyanins in seaweeds, but *Ceramium rubrum* Ag., *Griffithsia setacea* Ag., and *Plocamium coccineum* Syngb. contained leuco-compounds yielding pigments soluble in dilute acids and somewhat resembling betanin in reactions.

Senecio speciosus. Bright reddish mauve flowers; cyanidin diglycoside with a carbohydrate or other residue on hydroxyl in positions 3′ or 4′, the anthocyanin being Fe^{-}.

The cineraria, "Crimson Star," brownish bluish red; pelargonidin 3-bioside as previously reported for an unnamed variety.

Sutton's "Superb Crimson" cineraria was carefully studied and the anthocyanin is based on cyanidin only; its Fe^{-} property and diglycosidic nature again suggests substitution at 3′ or 4′.

Strelitza reginae. The orange petals contain no anthocyanin; the blue pistils contain delphinidin diglycoside, a little acylated pigment is present.

Streptocarpus Dunnii. Brownish pink flowers, cyanidin 3-pentoseglycoside. Dull salmon-pink flowers of another variety contained the same anthocyanin and much yellow pigment.

Symphyllum caucasicum. Blue flowers; delphinidin diglycoside.

Syncarpia laurifolia. The sawdust contains leuco-anthocyanin → cyanidin with some delphinidin.

Torenia Fourniers Grandiflora. Blue and violet flowers; cyanidin 3 : 5-dimonoside.

Tritoma speciosa. Orange-salmon flowers; pelargonidin 3-pentoseglycoside.

Tropaeolum majus. "Yellow Gleam" and "Golden Gleam" contain no anthocyanin. The double red-scarlet "Hermann Gnashoff" is coloured by pelargonidin 3-bioside.

Verbena. A range of hybrids has been examined, but the variety containing cyanidin dimonoside previously noted was not among them. Sutton's "Bedding Scarlet," "Firefly" and "Giant Pink" contained pelargonidin 3-monoside. "Purple White Eye" and "Bedding Blue" contain the same anthocyanin, the difference in colour, very large at a glance, is diminished on crushing the flowers and is due to local chemical or physical conditions to a large extent. The anthocyanin is a delphinidin saccharide with a high monoglycosidic type of distribution. It may be a complex diglycoside but certainly not of the usual type. The colour reactions fit in better with the 3-monoglycoside hypothesis. A series from an Oxford garden was arranged in order, most blue to most red. The violets and purples were all of the delphinidin type; there was no cyanidin, and the greatest surprise was the absence of pelargonidin 3-monoside. The rose-coloured varieties contained pelargonidin 3 : 5-dimonoside.

Viola. "La Grandeur," red-violet, complex delphinidin pentoseglycoside as anticipated but "Maggie Mott," blue, appears to be diglycosidic. "Archie Grant" seems to contain delphinidin pentoseglycoside free from acyl groups.

Vitis heterophylla humulifolia. The berries (at first colourless) change in colour from green to red to purple to purplish blue to turquoise-blue to yellow. The colourless and the green berries contain leuco-anthocyanin → cyanidin. This is definitely not pseudo-base as it requires vigorous treatment with hydrochloric acid in order to produce the anthocyanidin: the anthocyanin reaches a maximum in the purplish blue berries and it consists of malvidin 3 : 5-dimonoside.

Zea Mays var. *amylacea.* Dark blue and bright red cobs were procured from N.W. Arizona. The blue pigment appears as a fine powder in the starch protected by a cellulose sheath; the red cob contains the pigment more evenly distributed in all the structures. The blue pigment is easily soluble in 1 % hydrochloric acid and is cyanidin 3-monoside (evidently in neutral form or in colloidal combination). The red pigment is insoluble in 1 % hydrochloric acid but can be extracted by means of alcoholic hydrogen chloride; it is a complex derivative of pelargonidin.

Zinnia. "Fire King," bright pink-orange flowers; carotenoid and cyanidin 3 : 5-dimonoside.

The following experiments were made by Mr E. A. H. Roberts in collaboration with the authors.

The relation between co-pigment effects and distribution properties.

If an anthocyanin (m mg.) is distributed between V ml. each of equilibrated *iso*amyl alcohol and 0·5 % hydrochloric acid, D being the distribution number (% passing to the *iso*amyl alcohol), then if the anthocyanin is associated to double molecules in the aqueous layer, remaining unassociated in the *iso*amyl alcohol, we have $mD^2+kVD-100\,kV=0$, in which m and D are variables under standard experimental conditions. The condition laid down is satisfied if the curve $\log C_W/\log C_{AA}$ is linear with a slope of 2 and this is the case for oenin chloride and chrysanthemin chloride [Levy and Robinson, 1931]. Knowing D for some particular value of m, well inside the region of agreement with the formula, we can find the concentration at which the double molecules break up in the aqueous solution to a significant extent. We now estimate that this is about 2 mg. in 25 ml. of 0·5 % hydrochloric acid for oenin chloride and about double this concentration for both chrysanthemin and oxycoccicyanin chlorides; callistephin at such concentrations shows no association. More dilute solutions contain a considerable number of single molecules; more concentrated solutions contain double molecules and association to triple molecules is naturally a possibility, but has not been observed within the limits of our experiments.

The following supplementary determinations of D were made in the case of chrysanthemin: m, 1·32, D, 25·8; 2·1, 22·9; 2·9, 20·7; 3·6, 19·2; 5·6, 16·2.

Distribution numbers in the presence of papaverine or narcotine were determined by the standard method using 0·5 % hydrochloric acid containing the alkaloid (0·438 g. in 250 ml.). After the separation of the layers the solutions were made up to the same composition of solvents as usual, but they were also each saturated with papaverine or narcotine hydrochloride and compared at about the same concentration of pigment. Otherwise the co-pigment effect obscured the colorimetry. The results for oenin chloride and papaverine hydrochloride were: m, 1·53, D, 10·65; 2·02, 10·9; 2·96, 10·6; 3·3, 10·2; 4·56, 9·6; 5·7, 8·7. For chrysanthemin chloride and papaverine hydrochloride: m, 1·44, D, 17·9; 1·78, 16·7; 2·4, 15·0; 3·17, 14·1; 4·57, 13·7; 6·3, 13·7; 7·47, 12·3; 11·44, 12·5.

The effect of adding narcotine was relatively small (for oenin: m, 2·36, D, 10·2; 3·0, 18·2; 3·45, 18·0; 5·9, 14·0) and when the curves $\log C_W/\log C_{AA}$ are plotted it is found that over a considerable range the slope, with papaverine added, approximates to 1. Evidently the papaverine has broken up the double molecules probably by combining with the pigment, and as the alkaloid is in great excess this gives the effect of single molecules.

The slopes in the cases in which narcotine was introduced are approximately 2 and at no point so low as 1.

The effect of these addenda on the absorption spectra is shown in Fig. 1 for chrysanthemin (0·66 mg. in 25 ml. of 0·5 % hydrochloric acid), alone and with

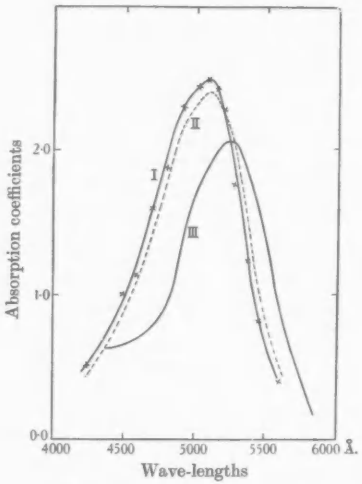

Fig. 1. I, chrysanthemin chloride; II, chrysanthemin chloride with narcotine; III, chrysanthemin chloride with papaverine.

the addition of papaverine hydrochloride (0·052 g.). The addition of narcotine (0·16 g.) produces a much smaller effect. Similar results were obtained with oenin chloride, the shift being in the same direction but smaller in magnitude; in this case the addition of papaverine slightly raised the position of the maximum.

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CCXXIX. TRYPTOPHAN DEFICIENCY.

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THE position of tryptophan is very much that of a vitamin; a substance—in this case of known constitution—essential to life, for the supply of which the animal is dependent upon outside sources. As with the vitamins, it is important that the reason for this indispensability should be understood.

The work on tryptophan function has followed three different lines. In the first place, the quantitative and qualitative aspects of the deficiency syndrome have been studied, and as no one has succeeded in measuring any effect other than the failure to grow, this work has resolved itself into following the influence of various factors on the weight curves of animals on diets low in tryptophan. An impressively large number of tryptophan derivatives has been given to such animals, and the only ones able to replace tryptophan itself would seem to be indolepyruvic acid [Jackson, 1929; Berg *et al.*, 1929, 1], *d*-indolelactic acid, but not the *l*-isomeride [Bauguess and Berg, 1934, 1], *d*-tryptophan [Berg and Potgieter, 1931], acetyl-*l*-tryptophan and its ethyl ester hydrochloride [Berg *et al.*, 1929, 2].

As indolepyruvic acid may be converted into tryptophan in the liver, and acetyltryptophan and the ester hydrochloride readily hydrolysed, it cannot be said that the above work has contributed anything of a really positive nature to the problem. An interesting observation is that of Berg and Rose [1929], who showed that feeding 20 mg. of tryptophan per rat at 24 hourly intervals gives submaximum growth, while 10 mg. doses at 12 hourly intervals give maximum growth. It would seem that the vital function is associated with one of the more fluid aspects of the animal's metabolism.

Du Vigneaud *et al.* [1932] report experiments in which tryptophan was injected into deficient animals with a consequent resumption of growth. A similar result was claimed by Schichiri and Sakata [1933], though it would seem from their figures that no very definite effect was obtained; the amounts of the amino-acid injected were never more than 20 % of the daily requirement, and in no case was a resumption of growth observed. The abnormally rapid fall in weight of the control animals would suggest that they were otherwise deficient.

Jackson [1927] reported negative results from tryptophan injections.

A second chapter in the tryptophan story deals with kynurenic acid formation. This has recently been reinvestigated by Kotake and his co-workers, who showed [Kotake and Iwao, 1931] that an intermediary substance, kynurenin, could be detected, and that the reaction is probably located in the liver [Ichihari *et al.*, 1931]. Kotake and Sakata [1931] would associate kynurenin with urochrome, although they ignore the obvious complexity of this group of substances, and their results manifestly refer to one fraction only.

Despite this and more recent work [Kotake, 1933], it can still be said of kynurenic acid that it appears to represent a shunt mechanism which is

available for dealing with an excess of tryptophan. Possibly it should be described as a detoxication mechanism.

Berg [1931] and Bauguess and Berg [1934, 2] have used this system as an index of tryptophan metabolism and have tested a number of substances for their convertibility into kynurenic acid. Among the substances replacing tryptophan in growth experiments they find that tryptophan ethyl ester hydrochloride, indolepyruvic and *dl*-indolelactic acids will, while acetyltryptophan and *d*-tryptophan will not, pass over into kynurenic acid. It seems most probable, however, that the only significance which can be attached to these results is that they may give a measure of the rate at which the substance in question may be transformed into indolepyruvic acid. From the work of Kotake and others, it seems likely that kynurenin and kynurenic acid are produced from tryptophan *via* the pyruvic acid, and any substance following this line of metabolism can be expected to be side-tracked to kynurenic acid only if it is likely, at any stage, to give rise to a large concentration of indolepyruvic acid or tryptophan itself. Thus the failure of acetyltryptophan might be due to the low rate of hydrolysis, and that of *d*-tryptophan to the fact that it is more slowly converted into indolepyruvic acid than the naturally occurring *l*-isomeride. Neither kynurenic acid [Assayama, 1916] nor kynurenin [Jackson and Jackson, 1932] will replace tryptophan in the diet.

Other researches into the function of tryptophan have been concerned with its haematopoietic effect. Hirazawa [1922] showed that anaemic animals recover the faster for tryptophan injections. Okagawa and Tatsui [1931] would associate this with the spleen, but their evidence does not seem adequate to support their conclusions. It has been shown here (unpublished work) that tryptophan is oxidised by peroxidases in the spleen, but the nature of the products has not yet been established, and the observation has in no way been correlated with the above findings.

Matsuoka and Nakao [1931], in demonstrating that 2-methyltryptophan, which will not replace dietary tryptophan, is efficacious in relieving anaemia, indicated that the haematopoietic effect is of a stimulatory nature, rather than the provision of a building-stone. In an earlier paper [Alcock, 1933] it is shown that tryptophan deficiency is not accompanied by anaemia, and that blood-formation can go on in the absence of dietary tryptophan. This would appear to dismiss the idea that the tryptophan is providing an essential constituent of the blood-protein, such as haematin.

Kynurenin has no haematopoietic effect [Iwakura *et al.*, 1933]. Fontés and Thivolle [1930] reported the relief of anaemias in human subjects by subcutaneous injection of a mixture of tryptophan and histidine, but this, together with the other findings reported in the same paper, has not been substantiated elsewhere.

From this mass of experimentation few positive conclusions can be drawn. Tryptophan is essential for growth and maintenance, and the need is immediate. Excess of tryptophan causes production of kynurenin and kynurenic acid, though no physiological significance has been attached to these substances. Injections of tryptophan will accelerate the recovery from anaemia, though such recovery is possible in the absence of either injected or dietary tryptophan. A further possible association is with the spleen, the reticulo-endothelial system generally and urochrome, but information on this point is meagre.

The really positive finding, and the one which is linked with tryptophan's essential nature, is the original one of Hopkins and Willcock [1906], that without this amino-acid animals decline and die. The other two series of observations would appear to be dealing with side-lines.

There are two possible causes for this decline on a tryptophan-deficient diet. It may be, and indeed it is usually assumed, that the animal is wholly unable to make tryptophan, and that therefore the fall in weight measures the rate of utilisation of the body proteins and of the tissue involved; the non-cyclopoietic animal being powerless to make good the loss. The other possibility is that tryptophan, or a derivative of it, exerts some specific effect upon the tissues or upon some one tissue—a "hormone" effect—entirely independent of the protein "Baustein" function mentioned above. The distinction between these two is important, not only in connection with tryptophan metabolism and significance, but also because it has a bearing upon the problem of the mode of protein synthesis *in vivo* and on the question of limiting factors to growth generally.

An attempt has therefore been made to decide to which of these the indispensable nature of tryptophan is to be ascribed. The preliminary work deals with the quantitative aspects of loss of weight in tryptophan deficiency. There follow experiments on the injection of (*a*) tryptophan and (*b*) the anterior pituitary growth hormone into tryptophan-deficient animals.

Experimental.

Most of the work on tryptophan deficiency has been carried out on rats, and the diet described by Berg and Rose [1929] has been almost universally adopted. The various workers have introduced slight variations into the composition of the diet, but nothing which would account for the wide variations in the results. Table I gives some idea of the falls in weight obtained by different workers using approximately the same conditions.

Table I.

Duration in days	Loss in g.	Reference
12	17	Matsuoka and Nakao [1931]
20	20	Berg *et al.* [1929, 2]
40	18	Jackson and Jackson [1932]
40	52	Schichiri and Sakata [1933]
40	35·5	See Table III

This wide variation is manifestly undesirable, as it becomes impossible to compare the results of one worker with those of another on a quantitative basis. In all cases there should be record of a control experiment where the diet itself has been supplemented with tryptophan; the "control" applied by some workers, a replacement of the hydrolysed caseinogen by unhydrolysed, is no control in effect.

The first need was clearly a tryptophan-deficient diet in which the tryptophan lack represented the limiting factor to growth, and which would give reasonably uniform results. It was felt that, if possible, the hydrolysed caseinogen should be replaced by a whole protein—the advantages are obvious; slower absorption, better taste, controlled blood-amino-acid concentration and so on. Gelatin is unsatisfactory and could not be used, even in conjunction with zein. Zein itself contains no lysine, and supplements of lysine would have proved expensive. However, a solution was found in various mixtures of zein and hydrolysed caseinogen.

The zein is prepared as follows. Maize gluten, obtainable from British Corn Products, Ltd., in a calico bag, is immersed in 80 % alcohol and allowed to soak for some hours. The bag is then allowed to drain, and the alcohol actually

soaked up by the gluten is squeezed out in a press. The squeezed gluten is again extracted, drained and pressed. Three or four such extractions can profitably be made. The solution of zein is then poured into a large volume of cold water, with vigorous stirring, and the suspension is allowed to settle overnight in the ice-chest. The zein is then picked out (by hand), placed in a muslin bag and rubbed through into a beaker of ice-cold water, from which it is allowed to settle. The water is decanted, the sludge of zein filtered on a Büchner funnel and spread out on large trays to dry. It may be necessary at this stage to grind it, but usually, if it has been kept cold, it dries as a fine powder. If at any time it sets into a dense rubbery mass, a few days in the ice-chest under water will render it friable and capable of being powdered through muslin. It should never be allowed to get warm. The yield is about 20 %.

The caseinogen is hydrolysed by three hours' treatment in an autoclave with 25 % sulphuric acid. This is removed with baryta, and the filtrates are evaporated in open pans and finally dried in a vacuum-oven.

In all the following experiments the vitamins were given in the form of three drops of cod-liver oil, and an alcoholic extract of autolysed yeast, the equivalent of 0·75 g. fresh yeast per rat daily. This preparation contains vanishingly small quantities of tryptophan, as does also the zein. The food was always given *ad lib.*, and from time to time, throughout the experiments, tests of intake were made. These gave a constant figure—between 6 and 8 g. per rat daily, with a tendency to a higher value on the better-tasting diet 25. This is probably set off by the relative indigestibility of the zein; in any case, the rates of growth on the supplemented diets do not differ markedly.

Three diets have been tested, the main difference being the relative proportions of hydrolysed to unhydrolysed protein. Diet 1 differs from that of Berg and Rose only in the slightly higher caseinogen content and the absence of a cystine supplement. This was found to make no difference.

Table II.

Constituents	Diet 1	Diet 15	Diet 25
Hydrolysed caseinogen	18	9	5
Zein	—	9	20
Rice starch	40	40	40
Sucrose	20	20	20
Palm kernel oil	17	17	10
Salt mixture	5	5	5

Table III summarises the results shown in Figs. 1, 2 and 3. It shows the average losses and gains on the different diets and the corresponding supplemented diets. The supplement consists of 0·25 % of tryptophan.

Table III.

Basal diets.	Average loss in weight, g.		
Duration in days	Diet 1	Diet 15	Diet 25
20	27·5	8·2	3·7
40	35·5	14·8	7·8
60	42·5	23·6	—
Supplemented diets.	Average gain in weight, g.		
20	20	26·5	21
40	45	43·5	49·5

The results given in Table III and Figs. 1, 2, 3 indicate that, although no great improvement in the growth rate on the supplemented diet could be

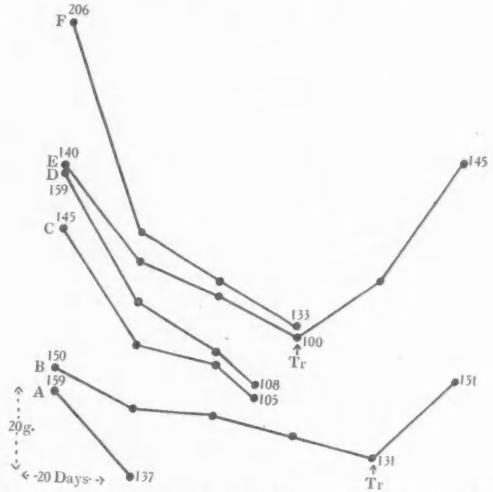

Fig. 1. Weight curves of rats on diet 1. At Tr ↑, B and E received tryptophan.

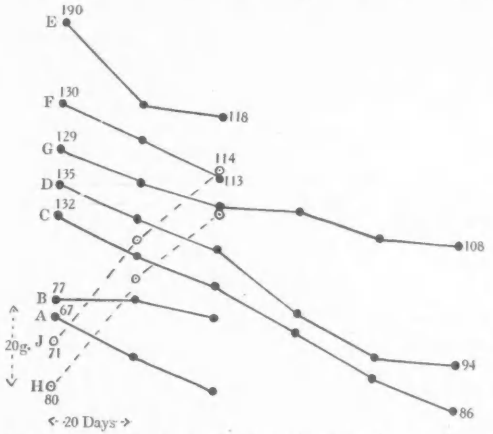

Fig. 2. Weight curves of rats on diet 15. Rats J and H received tryptophan.

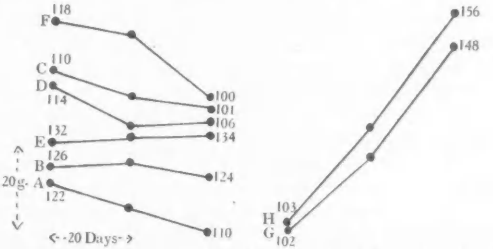

Fig. 3. Weight curves of rats on diet 25. Rats H and G received tryptophan.

achieved, the replacement of the hydrolysed protein by zein prevented the extensive losses in weight and kept the animals in much better condition. Blood counts for such animals are to be found in a previous paper [Alcock, 1933].

The almost negligible loss of weight on diet 25 would suggest that the tryptophan in proteins lies within the animal's synthetic powers. During the experimental period, the rats have been making some skin and hair, intestinal secretions, haemoglobin, hormones, *etc.*, involving appreciable amounts of tryptophan. To suggest that the amino-acids liberated from autolysed tissue proteins were utilised would imply an unbelievable economy and one which the results of Fürth and Lieben [1922] would make to appear all the more unlikely, as they showed that only 3 to 8% of ingested tryptophan was available for tissue building.

It is clear that if the limit to growth is imposed by the lack of tryptophan in the tissues for protein synthesis, then injections of this amino-acid will have the same positive effect upon the growth rate as that given by the mouth. The blood-concentration of free tryptophan will be restored, and protein building can continue. The work of Jackson [1927] suggests that this is not the case, but du Vigneaud *et al.* [1932] and Schichiri and Sakata [1933] both report the opposite result, that tryptophan injected under the skin is efficacious in relieving the symptoms of tryptophan deficiency.

The experiment has therefore been repeated here. Four rats were kept in the same cage and given tryptophan-deficient diet 25. Two were taken out daily and given a subcutaneous injection of 1 ml. of 2 % tryptophan solution, which had been filtered through a Seitz filter. The injections were continued for 15 days, and from the 20th day onward all four were given diet 25 with a supplement of 0·25 % tryptophan. It will be seen from Fig. 4 that the injections had no effect, although the animals responded normally to oral tryptophan subsequently.

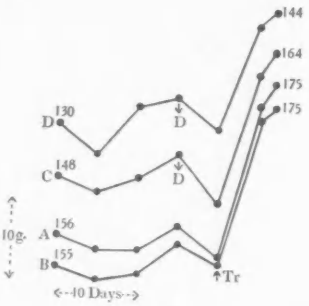

Fig. 4.

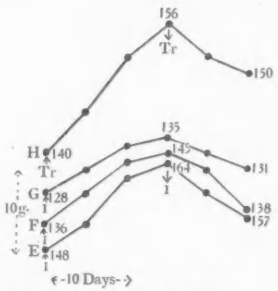

Fig. 5.

Fig. 4. Weight curves of rats on diets 25. C and D received daily injections of tryptophan to D ↓. At ↑Tr all four given tryptophan in the food.

Fig. 5. Rats on diet 25. G, F, and E received pituitary injections from I ↑ to I ↓, and H, tryptophan in the food to Tr ↓.

This suggests that the limit to growth is not imposed by the inability to make the tryptophan necessary for tissue proteins. If this is actually so, it might be possible to raise the growth limitation by other means than oral tryptophan, and thus get tissue production, with its local tryptophan synthesis, on a tryptophan-deficient diet.

This was accidentally arrived at in one experiment, in which a rat which had been placed on diet 25 was found to be pregnant—some 9 days gone, apparently—at the time of starting the experiment. Throughout the gestation, her weight did not alter in the least, and ultimately, she gave birth to a litter of six—small, and stillborn. The tissues of these rats had however been built up without any exogenous tryptophan.

In the course of a research on the question of the significance of growth limitation on deficient diets (Alcock and Mapson, to be published shortly), animals on diet 25 were given daily injections of a preparation of the anterior pituitary growth hormone [Evans and Simpson, 1931]. 0·1 to 0·15 ml. of this material was injected daily. The results are shown in Fig. 5, the three lower lines representing the rats so treated and the uppermost line a rat given 20 mg. of tryptophan by the mouth daily.

The weights of these three animals ran up steadily for the 15 days. After the injections had been discontinued, the weights fell off again, and the weight curves of the three injected animals closely paralleled that of the control. It is important to observe that there was no sudden falling off on the first day, but that the decline was gradual. This is significant, as the weight-increases resulting from such injections, in the normal animal, are due in part to water intake and in part to real growth, but while the growth effect remains, the whole of the excess water is excreted within the first 48 hours of the cessation of the injections. The shape of the curves makes it most probable that a real growth effect is involved, but the experimental period was too short to permit of any definite statement being made. The variations lie within the experimental error zone of nitrogen-balance determinations, and therefore these cannot be used to settle the matter.

An extension of the experiment to a longer period is made difficult by the sensitiveness of the skin of the deficient animals. Instead of becoming accustomed to the injections, as do normal animals, they become increasingly apprehensive as the time goes on, and seem to suffer real pain on the insertion of the needle, even though the injections are made at a different place on each occasion. It was this which also prevented an extension of the experimental period in the tryptophan injection work. The same effect was observed in animals suffering from vitamin deficiencies.

The evidence here presented would seem to justify the conclusion that the limiting factor to growth on a tryptophan-deficient diet does not arise from the demand of the tissues for amino-acids to be used for protein synthesis. There is no positive suggestion as to how the limitation can be accounted for, except that some material which is essential to life appears to be made in the liver (see experiments on tryptophan injection). This is probably not associated with kynurenic acid or kynurenin and is independent of the haematopoietic effects which tryptophan exerts when injected.

The fact, however, that the animal can synthesise proteins while receiving no tryptophan, means that, for this particular purpose it is able to make its own tryptophan. This carries with it the implication that the synthesis of protein probably starts at a lower stage than the fully formed amino-acid, and that the amino-acid groups actually found in the animal proteins have been formed there, not assimilated from the blood-stream as such. This brings the synthesis of protein in the animal into line with that in other organisms, in particular the plant, where the nitrogen source in protein building is necessarily of a simple nature.

It might be objected that, if the animal can make the tryptophan for its

proteins, it should be able to use that machinery for providing itself with what may be styled "essential," or hormonal tryptophan, when the normal supply of this fails. This, however, can be ruled out on two grounds; the quantitative and the teleological. On the latter, the need for such a transfer would never arise in nature; specific tryptophan deficiency is unknown. And on the former, the rat needs 20 mg. tryptophan daily—this means 1 g. of protein, or say 10 g. of tissue, which would have to be synthesised and autolysed during the course of 24 hours. Not only that, but it has been shown here that tryptophan delivered elsewhere than in the portal vein is not available for the "essential" purpose, and the autolytic tryptophan postulated above would be generally distributed.

So that, while it has not been possible to show why it is that tryptophan is essential to life, a study of the deficiency has thrown some light on protein synthesis, and the essential function has probably been identified as a prerogative of the liver.

SUMMARY.

1. A brief review of the knowledge of tryptophan function is given.
2. An improved tryptophan-deficient diet is described.
3. Rats on the above diet, injected with tryptophan, failed to grow.
4. Rats on the above diet gave a positive response to the anterior pituitary growth hormone and probably grew new tissues.

This work was carried out during the tenure of a Senior 1851 Studentship.

I would like to express my indebtedness to Sir F. G. Hopkins for his constant encouragement and advice.

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CCXXX. SYNTHESIS OF SUCROSE IN PLANT TISSUE.

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The transformations which different sugars undergo in plant tissue, independently of the photosynthetic process, are as yet imperfectly understood. There is every reason to assume that all the individual carbohydrates in plant material are capable of conversion into each other, though the experimental evidence at hand is insufficient to warrant definite conclusions in individual cases. This is also true of the most important hexose sugars, glucose and fructose. It is known that starch is readily formed from fructose when leaves that have been previously deprived of starch are kept in fructose solution in the dark. On the other hand, in wilting leaves starch is converted into sucrose. Similarly in the artichoke tuber, inulin—a condensation product of fructose—is in part converted into glucose [Colin, 1919]. It is also known that fructose is converted into glucose in the mammalian body, but evidence is still wanting to show whether a similar transformation takes place also in plants.

Although the formation of starch in leaves which are floated on solutions of various carbon compounds in the dark has been extensively studied by numerous workers, very little attention has been paid to other synthetic processes which possibly occur parallel with the synthesis of starch. In view of the very frequent occurrence of sucrose in various plant juices it was therefore of interest to study whether measurable amounts of sucrose were formed in leaves which were previously deprived of starch and then artificially fed with either glucose or fructose solutions in the dark. Such a synthesis would naturally involve a preliminary conversion of each of these hexose sugars into the other.

In fact, our experiments showed that sucrose is readily formed both from glucose and from fructose, the rate of synthesis being approximately equal in both cases.

In this connection it is interesting to note that Burkard and Neuberg [1934] have recently found in sugar-beet leaves phosphoric esters of both glucose and fructose.

Experimental.

Red clover and wheat were used as test plants as they produced a perfectly homogeneous material for analysis. Wheat proved to be the better of the two, owing to its more rapid growth and the fact that it did not contain appreciable amounts of reducing sugars. The plants were grown under greenhouse conditions in quartz sand in earthenware jars. Each jar contained about twenty wheat plants or four red clover plants. The experimental procedure was briefly as follows.

Red clover. An equal number of leaves was picked from each jar and the petioles were cut off. The leaves were rapidly chaffed, weighed and placed in a vacuum-drying apparatus which was previously heated to 100°. After 3 hours' drying *in vacuo* at 100° the material was weighed and ground to a powder, the dry matter content of which was determined from a small sample. After the leaves were picked, the jars were immediately placed in the dark for 24 hours in order to deprive the leaves of starch, whereupon an equal number of leaves was again picked from each jar and dried in the above manner. The remaining leaves were then cut off with their petioles and placed in a 10 % sugar solution for 24 hours in the dark. Care was taken that only the petioles came into contact with the sugar solution. For analysis, the petioles were cut off and the leaves were weighed, dried and ground to powder.

Wheat. The procedure followed was similar to that described above, except that three plants were taken from each jar, the leaves being cut for analysis. The sugar content of the leaves was determined again after 24 hours in the dark, whereupon the remaining plants were cut and placed in 10 % sugar solution so that the ends of the stalks reached some 5 cm. below the surface of the solution while the leaves did not come into direct contact with the liquid. After a further 24 hours in the dark the leaves were cut off from the stalks and analysed.

Estimation of sugars.

Total sugars. About 0·5 g. of the dry leaf-powder was weighed into a 150 ml. Erlenmeyer flask and hydrolysed on the water-bath with 30 ml. of 3 % HCl for 3 hours. The mixture was then filtered through asbestos, and the residue was washed repeatedly with small amounts of hot water, the volume of the liquid being made up to 100 ml. In this solution the total sugars were determined by Bertrand's method and calculated as glucose.

Soluble sugars. About 3 g. of the leaf-powder were weighed into a 150 ml. Erlenmeyer flask. 50 ml. of $M/15$ KH_2PO_4 solution were added, and the mixture was heated to boiling. After cooling, 0·5 ml. of toluene was added, and the flask was kept in an ice-chest overnight. The mixture was then filtered through asbestos, and the solution was made up to 100 ml. by repeated washing with hot water. The extract so obtained contained small amounts of protein which were removed with kaolin according to Michaelis and Rona [1908], and the liquid was centrifuged. Equal amounts of the protein-free extract were then taken for the estimation of (*a*) reducing sugars, (*b*) total soluble sugars, (*c*) sucrose. Reducing sugars were determined directly in aliquots of the solution by Bertrand's method and the total soluble sugars after hydrolysis by HCl on the water-bath for 15 minutes. For the estimation of sucrose an aliquot was treated with 0·5 ml. of toluene and 0·5 ml. of a concentrated invertase preparation[1]. The solution was kept at 32° for 1·5 hours, after which it was analysed for sugar according to Bertrand. The sucrose content of the solution was calculated as invert sugar from the differences in the amounts of Cu reduced by a given amount of the extract before and after the hydrolysis by the enzyme preparation. All values are given in percentages of dry matter.

Results.

Table I shows the results from an experiment in which 9 jars of red clover were used (May 7th, 1934).

[1] Invertinpräparat "Merck," No. 9098, supplied by E. Merck, Darmstadt. This preparation effects a complete inversion of sucrose in a few minutes, while maltose is not attacked.

Table I.

	Normal leaves	After 24 hours in the dark	After further 24 hours in 10 % glucose solution
Dry matter of the leaves, %	16·1	15·0	20·5
Reducing sugar	1·78	1·12	9·15
Sucrose	2·21	0·62	6·0

Other sugars were not determined. The results show that there is a striking accumulation of material in the leaves kept in glucose solution. The major part of the material is in the form of reducing sugar (probably glucose). There is, however, also a distinct formation of soluble, non-reducing sugar which is hydrolysed by invertase. 10·5 g. of dry leaf-powder were obtained from the leaves fed with glucose solution. Hence the amount of sucrose synthesised was 630 mg.

The following experiment was made with 240 wheat plants, cut about 2 weeks before blooming (May 23rd, 1934). The last portion of the leaves was divided into two lots, of which one was placed in 10 % glucose solution, the other in 10 % fructose solution.

Table II.

	Normal leaves	After 24 hours in the dark	After 24 hours in 10 % glucose solution	After 24 hours in 10 % fructose solution
Dry matter of the leaves, %	12·5	11·2	15·6	15·7
Total sugars	15·8	13·6	27·2	25·3
Total soluble sugars	3·65	Traces	15·7	15·0
Reducing sugar	Traces	0	9·1	8·25
Sucrose	3·52	0	6·35	6·0

It is particularly interesting to note that the whole amount of the synthesised, non-reducing sugar is hydrolysable by invertase, as shown by the fact that practically identical values were obtained when the extract of the powder was hydrolysed by HCl or by the invertase preparation. Thus, for instance, the following values were obtained for the leaf-powder in the experiment on fructose.

2·5 g. of the dry powder (dry matter 95·8 %) were extracted with KH_2PO_4 solution, and the volume of the extract was made up to 100 ml. as described above. 10 ml. of the protein-free extract, after hydrolysis by HCl, reduced 69·9 mg. Cu, corresponding to 35·9 mg. of glucose or 15·0 % of the dry matter. After hydrolysis by the invertase preparation, 10 ml. of the same extract reduced 68·9 mg. Cu, corresponding to 14·75 % of glucose in the dry matter. In the experiment with glucose the corresponding values were: 15·7 % after hydrolysis by HCl and 16·05 % after hydrolysis by invertase. The total amounts of sucrose synthesised in the above experiments were 775 mg. (from glucose) and 707 mg. (from fructose).

The results given in Table II also show that the synthesised sucrose actually originates from the simple hexoses (glucose and fructose, respectively) as the leaves contained only traces of soluble sugar when kept in the dark for 24 hours.

The fact that identical results were obtained on hydrolysis by HCl and by invertase also shows that the synthesised sucrose actually is present as such and not as a constituent of higher polysaccharides (raffinose, gentianose, *etc.*).

Other soluble disaccharides or polysaccharides were not formed. The above findings are best explained by assuming that a mutual conversion of glucose into fructose and *vice versa* takes place in plant tissue.

Research on the subject is being continued.

SUMMARY.

Sucrose is synthesised both from glucose and from fructose when leaves of red clover and wheat plants, previously kept in the dark for 24 hours, are placed in 10 % solutions of these hexoses for further 24 hours in the dark. In both cases the amount of sucrose synthesised was about 6 % of the dry matter of the leaves.

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CCXXXI. STUDIES ON THE ACTION OF EMULSIN. II.

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(*Received August 19th, 1934.*)

In the first paper in this series [Veibel and Bach, 1932] it was qualitatively shown that the difference in the rates of enzymic hydrolysis of the *d*-glucosides of the *d*- and *l*-components of optically active secondary alcohols, which has been examined mainly by Neuberg *et al.* [1929], does not disappear, even if the asymmetric carbon atom is not involved in the glucoside linkage. Thus of the two components of *dl*-3-methylpentyl-β-*d*-glucoside the *d*-alkyl-component is hydrolysed more quickly than the other.

The present paper deals with the extension of the work on the quantitative side and to glucosides prepared from *dl*-1-methylpentanol.

As an indication of the degree of hydrolysis, formerly determined by estimation of the amount of glucose set free, determination of the rotation of the reaction-mixture has been utilised, the enzyme action being stopped by addition of potassium carbonate. The rotations of the emulsin and the glucoside solutions were determined on other samples, and readings were taken to verify that the rotation of these solutions did not change during the experiment. All experiments were carried out at 30° and p_H 4·4.

Before starting the enzymic experiments the ratio of the two components in the glucoside mixture was determined by hydrolysis of the glucoside with acid, the liberated alcohol being isolated and its rotation determined.

Experiments were first made to determine whether the rate of hydrolysis, for a given concentration of glucoside, was proportional to the concentration of emulsin. The rates of hydrolysis of each component separately were determined by a second experiment with nearly the same concentration of glucoside as in the first, but with quantities large enough to permit of samples being withdrawn at different stages of the hydrolysis for examination of the liberated alcohol. From the reaction constants for the *d*- and the *l*-components determined in this way the apparent reaction constants for the first experiments were calculated and were found to correspond fairly well to the constants actually determined. In a further experiment the hydrolysis was allowed to proceed to the equilibrium-point and, in the same way as before, the reaction constants found by experiment were compared with those calculated.

It was found that for the 1-methylpentyl-β-*d*-glucoside the ratio k_d/k_l (k being the unimolecular reaction constant) was 3·44. For 3-methylpentyl-β-*d*-glucoside it was 1·07. In both cases proportionality was found between rate of hydrolysis and concentration of emulsin.

The comparison between experimentally found and calculated reaction constants is based upon an assumption of a linear relation between rotation of the reaction mixture and degree of hydrolysis. For 1-methylpentylglucoside

this does not hold good, owing to the great difference in rates of hydrolysis for the two components. The error involved in assuming a linear relation has been calculated and was found not to exceed 0·005°, *i.e.* the error is within the limit of experiment.

Shortly before my above-mentioned paper [1932] was printed, a very interesting paper by Mitchell and MacArthur [1932] appeared, in which the authors examined the rates of hydrolysis of the glucosides of some optically active alcohols, amongst others 1-methylpentanol. The reaction constants of the two components were determined by exponential analysis, assuming that the curve is of the form

$$y = a_1{}^{-k_1 t} + a_2{}^{-k_2 t},$$

where a_1, a_2, k_1, k_2 are constants, t is time and y is proportional to the concentration of unchanged glucoside. The hydrolysis was estimated by determining the amount of glucose set free, and the authors found the ratio $k_2/k_1 = 1{\cdot}5$. The method employed did not indicate whether the *d*- or the *l*-component were more quickly hydrolysed.

The great difference between this result and the result I have now obtained may be due to several causes. Mitchell and MacArthur employed concentrations, both of glucoside and of emulsin, far lower than those used by me (glucoside 0·0053 *M* against 0·04 *M*, emulsin 0·025 g. in 50 ml. against 0·2 g. in 50 ml.). I have not been able to try whether the concordance is better at such low concentrations, since my method does not allow a reliable control of reactions when the total change of rotation is only about 0·17°. Further, the English authors have possibly worked at a different p_H, since they seem to have dissolved glucoside and emulsin in water without addition of buffer.

Mitchell and MacArthur appear to estimate the ratio between the *d*- and the *l*-components in their glucoside mixture as 1, but they do not state whether they have verified this. They do not indicate the specific rotation of their glucoside tetra-acetate or of their glucoside but give analyses and melting-points only. Now it has been shown by Neuberg and co-workers, especially by von Falkenhausen and Neuberg [1931], in the first place that the synthesis starting from acetobromoglucose may proceed partially asymmetrically, and secondly that during the purification of the tetra-acetate and the glucoside itself by recrystallisation from the solvents commonly used the glucoside mixture may be partially or totally resolved into its components. Unpublished experiments by the author and Frederiksen have shown that the glucoside tetra-acetate of 1-methylpentanol is almost completely resolved by recrystallisation from diluted ethanol, so that a preparation of the acetate with melting-point 99° contains about 80 % *d*- and 20 % *l*-1-methylpentanol in glucosidic linkage, and a preparation of the glucoside with melting-point 115° contains 85–90 % *d*- and 15–10 % *l*-1-methylpentanol. If therefore Mitchell and MacArthur, as seems probable, used a glucoside mixture of 80–90 % *d*- and 20–10 % *l*-1-methylpentyl-β-*d*-glucoside, while estimating the ratio between the two components as 1 in their calculations, their results are decidedly open to question. In their Table II, where the agreement between experiment and calculation is shown, this possible error is however to some degree counterbalanced, since they start their calculations from the time 30 minutes, at which some 25 % of the glucoside has already been hydrolysed. The part first hydrolysed contains naturally most of the component of the highest reaction constant, which at the same time is the one most abundantly present, so that the remaining mixture at the time taken as zero by Mitchell and MacArthur may not differ so much from the mixture of equal parts of the two components as does the starting mixture. Further

examination may show whether this explanation of the difference between our results is correct or not.

For a given sample of emulsin, k/e (k=the unimolecular reaction constant, e=weight of emulsin in 50 ml. reaction mixture) may serve to compare the rates of hydrolysis of different glucosides, when in all cases the same (molar) concentration of glucoside is used. If different samples of emulsin are used, it is necessary to determine the enzymic power of each sample, *e.g.* indicated as sal. f. [Josephson, 1925; Helfferich, 1930; 1931]. In this case k/e (sal. f.) should make possible a comparison of results obtained with different samples of emulsin, but with glucoside solutions of the same molar concentration. In this way it seems possible to compare the influence of different alkyl groups on the rate of hydrolysis. The present investigation is to be continued in this direction.

Experimental.

I. *Standardisation of the emulsin preparation and experimental methods.*

The emulsin preparation was obtained from Merck and was used without further purification. Its enzymic power was determined from its action on salicin [Helfferich, 1930; 1931] and its sal. f. was found to be 0·045.

All experiments were carried out at $30 \pm 0{\cdot}1°$. The solutions were buffered with an acetate buffer; 100 ml. contained 10·0 ml. 0·1*N* CH_3COONa and 19·0 ml. 0·1*N* CH_3COOH; the p_H of such a solution (without glucoside and emulsin) was determined electrometrically at 30° to be 4·4.

The experiments were carried out in the following way.

Solutions of glucoside in water containing acetate buffer and of emulsin in water were placed in a thermostat at 30°. After adjustment of temperature calculated amounts of the two solutions were mixed in a measuring flask, which was then filled to the mark with water at 30°. In order to determine the progress of the hydrolysis samples of 5 ml. were withdrawn at different times and mixed with 1 ml. of 20 % potassium carbonate; after keeping for 3 hours to allow the mutarotation to take place, the rotation of the solution was determined in a 2 dm. tube, if necessary after filtration, and from the difference in rotation the degree of hydrolysis was calculated, the total variation in rotation having first been calculated from the initial rotation of the glucoside solution and the rotation of the resulting solution of glucose and alcohol.

All determinations of rotation were made with a polarimeter from Schmidt and Haensch, Berlin, with triple field, graduation in $\frac{1}{4}°$, vernier scale 0·01° so that 0·005° may be estimated. The source of light was an electric sodium lamp, also from Schmidt and Haensch, and the polarimeter was fitted with a monochromator, allowing the passage of pure D-light only. The polarimeter is a grant from the Carlsberg Foundation, to which my thanks are due.

II. *1-Methylpentyl-β-*d*-glucoside.*

(*a*) *Preparation and description.* The tetra-acetyl compound was prepared in the usual manner from acetobromoglucose, 1-methylpentanol and silver carbonate. The acetyl groups were removed, either with baryta as indicated by Fischer and Raske [1909], or with sodium methoxide according to Zemplén and Pascu [1929]. The two methods gave approximately the same yields.

The preparation here used was a mixture of the two glucosides corresponding to the *d*- and the *l*-components of the alcohol. The melting-point was, even after several recrystallisations from ethyl acetate, rather indefinite, 60–67°. The specific rotation of the preparation, which was used for all experiments

here recorded, was $[\alpha]_D^{19} = -32{\cdot}50°$ (water: $c = 3{\cdot}323$, $l = 2$). (Found: C, 54·84; H, 8·95 %. $C_{12}H_{24}O_6$ requires C, 54·50; H, 9·16 %.)

3 g. of the glucoside were boiled for 2 hours with 200 ml. water and 25 ml. 4*N* sulphuric acid. The alcohol set free was then distilled off with the first 100 ml. distillate. The distillate was extracted with ether, the ethereal solution dried over potassium carbonate, the ether distilled off and the residue fractionated. The fraction with B.P. 135–40° was regarded as pure l-methylpentanol. Its rotation in a 1 dm. tube was +1·05°; the density being 0·823, $[\alpha]_D^{19°}$ is found to be +1·28°.

Pickard and Kenyon [1911] give for the optically pure compound $[\alpha]_D = \pm 11{\cdot}57°$; a preparation with $[\alpha]_D = +1{\cdot}28°$ contains therefore 55·5 % *d*- and 44·5 % *l*-compound.

As mentioned above, the glucoside tetra-acetate is partially resolved into its two components by recrystallisation from dilute alcohol. The examination of the degree of this resolution is being continued.

(*b*) *Proportionality between concentration of emulsin and rate of hydrolysis.* A solution of glucoside in buffer solution was prepared which contained 2·6278 g. glucoside in 100 ml., and a solution of emulsin in water which contained 0·0719 g. in 10 ml. The emulsin solution was made by shaking 1 g. of emulsin thoroughly with water, filtering the mixture and filling up to 100 ml. in a measuring flask. The solid content of the solution was determined by evaporating 10 ml. to dryness at 105°.

Mixtures as indicated in Tables I–V were then prepared in measuring flasks of 50 ml. and samples of 5 ml. were withdrawn at times stated in the tables. The samples were mixed with 1 ml. 20 % potassium carbonate and their rotations determined. The emulsin solution had a rotation of −0·13° during the whole of the experiment, the glucoside solution a rotation of −0·57°. From this value the rotation of the solution after complete hydrolysis is calculated to +0·64°, taking into account that the l-methylpentanol present has a specific rotation of +1·28°.

Table I.

10 ml. emulsin solution, water + buffer to 50 ml.
$c_{emulsin} = 0{\cdot}0719$ g. in 50 ml., $c_{glucoside} = 0$.

Time min.	α
5	−0·13
50	−0·13
100	−0·14
1500	−0·13

Table II.

20 ml. glucoside solution, water to 50 ml.
$c_{emulsin} = 0$, $c_{glucoside} = 0{\cdot}0398\ M$.

Time min.	α
5	−0·56
50	−0·57
100	−0·57
1500	−0·57

Table III.

10 ml. emulsin solution, 20 ml. glucoside solution, water to 50 ml.,
$c_{emulsin} = 0{\cdot}0719$ g. in 50 ml., $c_{glucoside} = 0{\cdot}0398\ M$.

Time min.	α	$\alpha_{gluc.}$	x	$c - x$	$k \cdot 10^4$	%
0·00	−0·70	−0·57	—	1·21	—	—
9·97	−0·58	−0·45	0·12	1·09	45·5	9·9
20·00	−0·49	−0·36	0·21	1·00	41·4	17·4
40·00	−0·31	−0·18	0·39	0·82	42·3	32·2
60·00	−0·18	−0·05	0·52	0·69	40·7	43·0
80·00	−0·08	+0·05	0·62	0·59	39·0	51·2
110·00	+0·05	+0·18	0·75	0·46	38·2	62·0
150·00	+0·15	+0·28	0·85	0·36	35·1	70·2

Table IV.

20 ml. emulsin solution, 20 ml. glucoside solution, water to 50 ml.
$c_{emulsin} = 0{\cdot}1438$ g. in 50 ml., $c_{glucoside} = 0{\cdot}0398\ M$.

Time min.	α	$\alpha_{gluc.}$	x	$c-x$	$k.10^4$	%
0·00	−0·83	−0·57	—	1·21	—	—
10·00	−0·62	−0·36	0·21	1·00	82·8	17·4
20·00	−0·46	−0·20	0·37	0·84	79·3	30·8
39·97	−0·22	+0·04	0·61	0·60	76·2	50·4
60·00	−0·06	+0·20	0·77	0·44	73·2	63·6
80·00	+0·04	+0·30	0·87	0·34	68·9	71·9
110·00	+0·13	+0·39	0·96	0·25	62·3	79·2
150·00	+0·20	+0·46	1·03	0·18	55·2	85·1

Table V.

30 ml. emulsin solution, 20 ml. glucoside solution.
$c_{emulsin} = 0{\cdot}2157$ g. in 50 ml., $c_{glucoside} = 0{\cdot}0398\ M$.

Time min.	α	$\alpha_{gluc.}$	x	$c-x$	$k.10^4$	%
0·00	−0·96	−0·57	—	1·21	—	—
10·00	−0·66	−0·27	0·30	0·91	123·8	24·8
20·00	−0·44	−0·05	0·52	0·69	122·0	43·0
40·00	−0·18	+0·21	0·78	0·43	112·3	64·5
60·00	−0·04	+0·35	0·92	0·29	103·4	76·1
80·00	+0·06	+0·45	1·02	0·19	100·5	84·3
110·00	+0·12	+0·51	1·08	0·13	88·1	89·3
150·00	+0·16	+0·55	1·12	0·09	75·2	92·6

From the tables it is seen that the unimolecular reaction constants (which are all calculated with logarithms to base 10 and the minute as unit of time) fall steadily at all concentrations of emulsin. This may indicate that the two components of the glucoside are hydrolysed with rather different velocities.

The initial values of the constants show that there is a fairly good proportionality between concentration of emulsin and velocity of hydrolysis, the value of k/e being about $5{\cdot}8.10^{-2}$.

(*c*) *Determination of the rates of hydrolysis of the two components.* In order to find the reaction constants of the two components the following experiment was made:

15 g. glucoside were dissolved in 600 ml. acetate-buffer and mixed with 900 ml. emulsin solution, both solutions having been brought to 30°. The concentration of glucoside in the resulting mixture was $0{\cdot}0391\ M$, the rotation of the glucoside was −0·560° at the beginning, +0·625° after complete hydrolysis. The concentration of the emulsin solution was 0·2067 g. in 50 ml., the rotation of the emulsin present in the solution was −0·375°. The velocity constant was approximately calculated from the relation $k/e = 5{\cdot}8.10^{-2}$ and the times necessary to bring about a hydrolysis of 25 %, 40 % and 65 % respectively were calculated. At times determined in this way samples of 5 ml. were withdrawn and treated as usual, and 1 minute after each sample of 5 ml. samples large enough to contain about 2 g. hydrolysed glucoside were taken; the enzyme action in these samples was stopped by addition of a concentrated potassium carbonate solution, the alcohol set free was isolated in the way described above, collected in 5 ml. measuring flasks and filled up to the mark with absolute alcohol, and then the rotation was determined. The specific rotation of the optically pure

component, which is 11·57° without solvent, was determined in absolute alcoholic solution and found to be 12·48° ($c=10{\cdot}328$, $l=2$). Consequently the percentage content of *d*-component in a certain solution is calculated from the equation

$$12{\cdot}48.d-12{\cdot}48\,(100-d)=\alpha.100,$$

α being the experimentally found specific rotation.

The real degree of hydrolysis of the large samples is calculated from the foregoing 5 ml. sample, assuming that the reaction constant does not alter sensibly during 1 minute.

The results are given in Tables VI and VII. The figures in heavy type in Table VII are the calculated figures.

Table VI.

Rotation of the alcohol isolated.

10·95 min. $\alpha^{15°}=+1{\cdot}12°$, $c=7{\cdot}756$, $l=2$, $[\alpha]_D^{15°}=+7{\cdot}22°$.
22·00 min. $\alpha^{19°}=+0{\cdot}87°$, $c=6{\cdot}780$, $l=2$, $[\alpha]_D^{19°}=+6{\cdot}62°$.
45·00 min. $\alpha^{15°}=+1{\cdot}09°$, $c=9{\cdot}730$, $l=2$, $[\alpha]_D^{19°}=+5{\cdot}60°$.

Table VII.

$c_{\text{emulsin}}=0{\cdot}2067$ g. in 50 ml., $c_{\text{glucoside}}=0{\cdot}0391$ *M*.

		Time min.	α	$\alpha_{\text{gluc.}}$	x	$c-x$	$k.10^4$	%	Containing % *d*	Containing % *l*
Mixed Sample	5 ml.	0·00	−0·935	−0·560	—	1·185	—	—	—	—
,,	710 ,,	10·00	−0·650	−0·275	0·285	0·900	119·5	24·1		
,,	710 ,,	10·95	—	—	**0·308**	**0·877**	**119·5**	**26·0**	78·9	21·1
,,	5 ,,	21·00	−0·445	−0·070	0·490	0·695	110·4	41·4		
,,	460 ,,	22·00	—	—	**0·508**	**0·677**	**110·4**	**42·9**	75·7	24·3
,,	5 ,,	44·00	−0·190	+0·185	0·745	0·440	97·8	62·9		
,,	310 ,,	45·00	—	—	**0·755**	**0·430**	**97·8**	**63·7**	72·4	27·6

It is now possible to calculate the concentration of either component at the time at which the samples were taken and thus to calculate the two velocity constants. This is done in Table VIII.

Table VIII.

Time min.	x_d	x_l	c_d-x_d	c_l-x_l	$k_d.10^4$	$k_l.10^4$	k_d/k_l
0	—	—	0·0217	0·0174	—	—	—
10·95	0·0080	0·0021	0·0137	0·0153	182·4	51·0	3·46
22·00	0·0127	0·0041	0·0090	0·0133	173·7	53·1	3·31
45·00	0·0180	0·0069	0·0037	0·0105	170·7	48·8	3·55
				Mean values	175·6	51·0	3·44

In order to use these constants for controlling experiments with other emulsin concentrations it is necessary to calculate the quotients k/e, e being the emulsin concentration indicated as g. emulsin in 50 ml. reaction mixture, here 0·2067.

We thus get $k_d/e=8{\cdot}50.10^{-2}$, $k_l/e=2{\cdot}47.10^{-2}$.

In Tables IX–XI the experiments described above in part (*b*) are controlled in this way. It may be seen that the values calculated agree tolerably well with those found, the deviation in α being only in one case as much as 0·03°, in most cases 0·01° or less.

Table IX.

c_d=0·0221, k_d=61·1.10^{-4}, c_l=0·0177, k_l=17·9.10^{-4}, e=0·0719.

Time min.	c_d-x_d	x_d	c_l-x_l	x_l	x_{tot}	$c-x_{tot.}$	$k.10^4$ calc.	$k.10^4$ f.	% calc.	% f.	α calc.	α f.
0	0·0221	—	0·0177	—	—	0·0398	—	—	—	—	−0·57	−0·57
10	0·0192	0·0029	0·0170	0·0007	0·0036	0·0362	41·2	45·5	9·1	9·9	−0·46	−0·45
20	0·0167	0·0054	0·0163	0·0014	0·0068	0·0330	40·7	41·4	17·1	17·4	−0·36	−0·36
40	0·0126	0·0095	0·0150	0·0027	0·0122	0·0276	39·7	42·3	30·7	32·2	−0·20	−0·18
60	0·0093	0·0126	0·0138	0·0039	0·0165	0·0233	38·8	40·7	41·5	43·0	−0·07	−0·05
80	0·0072	0·0149	0·0127	0·0050	0·0199	0·0199	37·6	39·0	50·0	51·2	+0·04	+0·05
110	0·0047	0·0174	0·0112	0·0065	0·0239	0·0159	36·2	38·2	60·1	62·0	+0·16	+0·18
150	0·0027	0·0194	0·0095	0·0082	0·0276	0·0122	34·2	35·1	69·4	70·2	+0·27	+0·28

Table X.

c_d=0·0221, k_d=122·2.10^{-4}, c_l=0·0177, k_l=35·7.10^{-4}, e=0·1438.

Time min.	c_d-x_d	x_d	c_l-x_l	x_l	$x_{tot.}$	$c-x_{tot.}$	$k.10^4$ calc.	$k.10^4$ f.	% calc.	% f.	α calc.	α f.
0	0·0221	—	0·0177	—	—	0·0398	—	—	—	—	−0·57	−0·57
10	0·0167	0·0054	0·0163	0·0014	0·0068	0·0330	81·4	82·8	17·1	17·4	−0·36	−0·36
20	0·0126	0·0095	0·0150	0·0027	0·0122	0·0276	79·5	79·3	30·7	30·6	−0·20	−0·20
40	0·0072	0·0149	0·0127	0·0050	0·0199	0·0199	75·3	76·2	50·0	50·4	+0·04	+0·04
60	0·0041	0·0180	0·0108	0·0069	0·0249	0·0149	71·1	73·2	62·6	63·6	+0·19	+0·20
80	0·0023	0·0198	0·0092	0·0085	0·0283	0·0115	67·4	68·9	71·1	71·9	+0·29	+0·30
110	0·0010	0·0211	0·0072	0·0105	0·0316	0·0082	62·4	62·3	79·4	79·2	+0·39	+0·39
150	0·0003	0·0218	0·0052	0·0125	0·0343	0·0055	57·3	55·2	86·2	85·1	+0·47	+0·46

Table XI.

c_d=0·0221, k_d=183·4.10^{-4}, c_l=0·0177, k_l=53·6.10^{-4}, e=0·2157.

Time min.	c_d-x_d	x_d	c_l-x_l	x_l	$x_{tot.}$	$c-x_{tot.}$	$k.10^4$ calc.	$k.10^4$ f.	% calc.	% f.	α calc.	α f.
0	0·0221	—	0·0177	—	—	0·0398	—	—	—	—	−0·57	−0·57
10	0·0145	0·0076	0·0156	0·0021	0·0097	0·0301	121·3	123·8	24·4	24·8	−0·27	−0·27
20	0·0095	0·0126	0·0138	0·0039	0·0165	0·0233	116·3	122·0	41·5	43·0	−0·07	−0·05
40	0·0041	0·0180	0·0108	0·0069	0·0249	0·0149	106·7	112·3	62·6	64·5	+0·19	+0·21
60	0·0018	0·0203	0·0084	0·0093	0·0296	0·0102	98·6	103·4	74·4	76·1	+0·33	+0·35
80	0·0008	0·0213	0·0066	0·0111	0·0324	0·0074	91·3	100·5	81·4	84·3	+0·42	+0·45
110	0·0002	0·0219	0·0046	0·0131	0·0350	0·0048	83·5	88·1	87·9	89·3	+0·49	+0·51
150	0·0000	0·0221	0·0028	0·0149	0·0370	0·0028	76·9	75·2	93·0	92·6	+0·56	+0·55

(*d*) *Determination of the equilibrium-point.* In an experiment with approximately the same emulsin and glucoside concentrations as in part (*c*) the hydrolysis was followed during 480 minutes. An additional reading was made after 1440 minutes. Table XII gives the result, which is that the equilibrium-point is at some 95 % hydrolysis.

Table XIII gives a comparison of the calculated and the experimentally found values of k, degree of hydrolysis and α. The calculation is made in the same way as in Tables IX–XI. $c_{glucoside}$ is here 0·0406 M, $c_{emulsin}$ is 0·2006 g. in 50 ml., k_d is calculated to be 170·5.10^{-4}, k_l to be 49·5.10^{-4}.

A calculation of α like the one here given is based upon the assumption of a linear relation between rotation of the reaction mixture and degree of hydrolysis. As the relation k_d/k_l has been found to be 3·44, this assumption does not hold good. In order to make an exact calculation it is necessary to know the specific rotations of the two glucoside components. These rotations are not yet known, but unpublished experiments (by the author and Frederiksen) make it seem probable that they are not far from −29·5° and −36·7° respectively. Since the difference between the figures calculated with these values as base

Table XII.

$c_{emulsin}$ = 0·2006 g. in 50 ml., $\alpha_{emulsin}$ = −0·365°.
$c_{glucoside}$ = 0·0406 *M*, $\alpha_{beginning}$ = −0·580°, α_{end} = +0·645°.

Time min.	α	$\alpha_{corr.}$	x	$c-x$	$k.10^4$	%
0·0	−0·945	−0·580	—	1·225	—	—
5·0	−0·800	−0·435	0·145	1·080	109·4	11·8
10·0	−0·670	−0·305	0·275	0·950	110·5	22·4
20·0	−0·480	−0·115	0·465	0·760	103·7	38·0
40·0	−0·215	+0·150	0·730	0·495	98·4	59·6
60·0	−0·085	+0·280	0·860	0·365	87·6	70·2
80·0	+0·020	+0·385	0·965	0·260	84·2	78·8
100·0	+0·070	+0·435	1·015	0·210	76·6	82·9
120·0	+0·105	+0·470	1·050	0·175	70·4	85·7
140·0	+0·135	+0·500	1·080	0·145	66·2	88·2
160·0	+0·175	+0·540	1·120	0·105	66·7	91·4
190·0	+0·195	+0·560	1·140	0·085	61·0	93·1
220·0	+0·185	+0·550	1·130	0·095	—	92·2
250·0	+0·200	+0·565	1·145	0·080	—	93·5
280·0	+0·200	+0·565	1·145	0·080	—	93·5
310·0	+0·200	+0·565	1·145	0·080	—	93·5
360·0	+0·205	+0·570	1·150	0·075	—	93·9
420·0	+0·210	+0·575	1·155	0·070	—	94·3
480·0	+0·220	+0·585	1·165	0·060	—	95·1
1440·0	+0·220	+0·585	1·165	0·060	—	95·1

Table XIII.

c_d = 0·0225, k_d = 170·5.10⁻⁴, c_l = 0·0181, k_l = 49·5.10⁻⁴, e = 0·2006.

Time min.	c_d-x_d	x_d	c_l-x_l	x_l	$x_{int.}$	$c-x_{tot.}$	$k.10^4$ calc.	$k.10^4$ f.	% calc.	% f.	α calc.	α f.
0	0·0225	—	0·0181	—	—	0·0406	—	—	—	—	−0·580	−0·580
5	0·0185	0·0040	0·0171	0·0010	0·0050	0·0356	114·2	109·4	12·3	11·8	−0·430	−0·435
10	0·0152	0·0073	0·0162	0·0019	0·0092	0·0314	111·6	110·5	22·7	22·4	−0·300	−0·305
20	0·0103	0·0122	0·0144	0·0037	0·0159	0·0247	107·9	103·7	39·2	38·0	−0·100	−0·115
40	0·0047	0·0178	0·0115	0·0066	0·0244	0·0162	99·8	98·4	60·1	59·6	+0·155	+0·150
60	0·0021	0·0204	0·0091	0·0090	0·0294	0·0112	93·2	87·6	72·4	70·2	+0·305	+0·280
80	0·0010	0·0215	0·0073	0·0108	0·0323	0·0083	86·2	84·2	79·6	78·8	+0·395	+0·385
100	0·0004	0·0221	0·0058	0·0123	0·0344	0·0062	81·6	76·6	84·7	82·9	+0·460	+0·435
120	0·0002	0·0223	0·0046	0·0135	0·0358	0·0048	77·3	70·4	88·2	85·7	+0·500	+0·470
140	0·0001	0·0224	0·0037	0·0144	0·0368	0·0038	73·5	66·2	90·6	88·2	+0·530	+0·500
160	0·0000	0·0225	0·0029	0·0152	0·0377	0·0029	71·6	66·7	92·9	91·4	+0·555	+0·540
190	0·0000	0·0225	0·0021	0·0160	0·0385	0·0021	67·7	61·0	94·8	93·1	+0·580	+0·560

and those calculated assuming a linear relation does not exceed 0·005°, which is within the experimental limits, I have given the "linear" figures only.

It will be seen from Table XIII that up to some 70 % hydrolysis the agreement between the calculated and the found values of α is excellent. The agreement between calculated and found values of k and degree of hydrolysis is not quite as good; this is due to the fact that the found values are calculated from the found values of α and, owing to the deviation from a linear relation mentioned above, the real degree of hydrolysis is somewhat greater than that calculated from α.

III. *3-Methylpentyl-β-*d*-glucoside.*

(*a*) *Preparation and description.* The glucoside was prepared as previously described [Veibel and Bach, 1932], the acetyl groups however being removed as indicated by Zemplén and Pascu [1929]. (Found: C, 54·20; H, 9·16 %. $C_{12}H_{24}O_6$ requires C, 54·50; H, 9·16 %.) The melting-point was 83–88°, the specific rotation −30·85° (water, c = 2·269, l = 2).

2·5 g. glucoside were unspecifically hydrolysed and yielded 0·3304 g. alcohol with B.P. 145–51°, which, dissolved in absolute alcohol to 5 ml., gave a rotation of −0·015°, whence $[\alpha]_D = -0{\cdot}111°$. $[\alpha]_D$ for the optically pure compound being +8·77° [Hardin, 1908], this means that the content of *d*-component is 49·37 %, of *l*-component 50·63 %.

(*b*) *Proportionality between concentration of emulsin and rate of hydrolysis.* The experiments were made in exactly the same manner as indicated above for the 1-methylpentylglucoside. The reaction mixtures had the composition indicated in Tables XIV–XVIII. The emulsin solution had a rotation of −0·13°, the glucoside solution a rotation of −0·540°. From the latter value the rotation of the solution after complete hydrolysis is calculated to be +0·625°, the rotation of the alcohol set free being without measurable importance.

It will be seen that, apart from the initial values, the unimolecular reaction constants are real constants, and that there is sufficient evidence for the proportionality between the concentration of emulsin and the rate of hydrolysis,

Table XIV.

10 ml. emulsin solution, water + buffer to 50 ml.
$c_{\text{emulsin}} = 0{\cdot}0712$ g. in 50 ml.,
$c_{\text{glucoside}} = 0$.

Time min.	α
0	−0·130
160	−0·130
1620	−0·130

Table XV.

20 ml. glucoside solution, water to 50 ml.
$c_{\text{emulsin}} = 0$.
$c_{\text{glucoside}} = 0{\cdot}0398\ M$.

Time min.	α
0	−0·540
160	−0·540
1620	−0·540

Table XVI.

10 ml. emulsin solution, 20 ml. glucoside solution, water to 50 ml.
$c_{\text{emulsin}} = 0{\cdot}0712$ g. in 50 ml., $c_{\text{glucoside}} = 0{\cdot}0398\ M$.

Time min.	α	$\alpha_{\text{gluc.}}$	x	$c-x$	$k.10^4$	%
0·00	−0·670	−0·540	—	1·165	—	—
5·15	−0·660	−0·530	0·010	1·155	7·3	0·9
10·00	−0·645	−0·515	0·025	1·140	9·4	2·2
20·00	−0·630	−0·500	0·040	1·125	7·6	3·4
40·00	−0·600	−0·470	0·070	1·095	6·7	6·0
60·00	−0·570	−0·440	0·100	1·065	6·5	8·6
80·00	−0·550	−0·420	0·120	1·045	5·9	10·3
110·00	−0·485	−0·355	0·185	0·980	6·8	15·9
150·00	−0·450	−0·320	0·220	0·945	6·1	18·9
1620·00	+0·395	+0·525	1·065	0·100	6·6	91·4

Table XVII.

20 ml. emulsin solution, 20 ml. glucoside solution, water to 50 ml.
$c_{\text{emulsin}} = 0{\cdot}1424$ g. in 50 ml., $c_{\text{glucoside}} = 0{\cdot}0398\ M$.

Time min.	α	$\alpha_{\text{gluc.}}$	x	$c-x$	$k.10^4$	%
0·00	−0·800	−0·540	—	1·165	—	—
5·00	−0·775	−0·515	0·025	1·140	18·9	2·2
10·00	−0·745	−0·485	0·055	1·110	21·0	4·7
20·00	−0·715	−0·455	0·085	1·080	16·5	7·3
40·00	−0·660	−0·400	0·140	1·025	13·9	12·0
60·00	−0·610	−0·350	0·190	0·975	12·9	16·3
80·00	−0·540	−0·280	0·260	0·905	13·7	22·3
110·00	−0·480	−0·220	0·320	0·845	12·7	27·5
150·00	−0·400	−0·140	0·400	0·765	12·2	34·3
1620·00	+0·320	+0·580	1·120	0·045	—	96·1

Table XVIII.

30 ml. emulsin solution, 20 ml. glucoside solution.
$c_{emulsin} = 0{\cdot}2136$ g. in 50 ml., $c_{glucoside} = 0{\cdot}0398\ M$.

Time min.	α	$\alpha_{gluc.}$	x	$c-x$	$k.10^4$	%
0·00	−0·930	−0·540	—	1·165	—	—
5·00	−0·875	−0·485	0·055	1·110	42·0	4·7
10·00	−0·850	−0·460	0·080	1·085	30·9	6·9
20·00	−0·805	−0·415	0·125	1·040	24·7	10·7
40·00	−0·720	−0·330	0·210	0·955	21·6	18·0
60·00	−0·650	−0·260	0·280	0·885	19·9	24·0
80·00	−0·575	−0·185	0·355	0·810	19·7	30·5
110·00	−0·500	−0·110	0·430	0·735	18·2	36·9
150·00	−0·380	+0·010	0·550	0·615	18·5	47·2
1620·00	+0·200	+0·590	1·130	0·035	—	97·0

which is in this case only 1/6 to 1/7 of the rate of hydrolysis of 1-methylpentyl-glucoside, k/e being only about $0{\cdot}9.10^{-2}$ against $5{\cdot}8.10^{-2}$.

(*c*) *Determination of the rates of hydrolysis of the two components.* From the constancy of the reaction constants determined above it seems likely that the two components of the glucoside are hydrolysed with nearly the same velocity. In order to determine the two reaction constants the following experiment was made.

10 g. glucoside were dissolved in 500 ml. acetate buffer and mixed with 500 ml. emulsin solution, both solutions having been brought to 30°. The concentration of glucoside in the resulting mixture was $0{\cdot}0398\ M$, the rotation of the glucoside was −0·540° at the beginning, +0·625° after complete hydrolysis. The concentration of emulsin was 0·1829 g. in 50 ml., the rotation of the emulsin was −0·325°. The velocity constant was determined by interpolation to be approximately 16.10^{-4}, and the times necessary for bringing about a hydrolysis of 40 % and 60 % respectively were calculated. As in the foregoing experiment small samples were taken in order to control the degree of hydrolysis and larger samples to isolate the alcohol set free in order to determine how much of each component it contained. Tables XIX and XX give the results.

Table XIX.

Rotation of the alcohol isolated.

136 min. $\alpha^{19°} = +0{\cdot}015°$, $c = 6{\cdot}418$, $l = 2$, $[\alpha]_D^{19°} = +0{\cdot}117°$.
243 min. $\alpha^{19°} = \pm 0°$, $[\alpha]_D^{19°} = \pm 0°$.

Table XX.

$c_{emulsin} = 0{\cdot}1829$ g. in 50 ml., $c_{glucoside} = 0{\cdot}0398\ M$.

		Time min.	α	$\alpha_{gluc.}$	x	$c-x$	$k.10^4$	%	Containing % d	Containing % l
Mixed Sample	5 ml.	0	−0·865	−0·540	—	1·165	—	—	—	—
,,	590 ,,	135	−0·420	−0·095	0·445	0·720	15·45	38·20		
,,	590 ,,	136	—	—	**0·447**	**0·718**	**15·45**	**38·37**	50·67	49·33
,,	5 ,,	242	−0·200	+0·125	0·665	0·500	15·18	57·08		
,,	390 ,,	243	—	—	**0·667**	**0·498**	**15·18**	**57·25**	50·00	50·00

The calculated values of the concentration of either component and of k_d and k_l are given in Table XXI.

Table XXI.

Time min.	$c_d - x_d$	x_d	$c_l - x_l$	x_l	$k_d . 10^4$	$k_l . 10^4$	k_d/k_l
0	0·0196	—	0·0202	—	—	—	—
136	0·0118	0·0078	0·0127	0·0075	16·2	14·8	1·09
243	0·0082	0·0114	0·0088	0·0114	15·6	14·8	1·05
				Mean values	15·9	14·8	1·07

e being 0·1829, we get $k_d/e = 0{\cdot}869 . 10^{-2}$, $k_l/e = 0{\cdot}809 . 10^{-2}$. With these values k_d and k_l in the experiments described in Tables XVI–XVIII are calculated and the found values of k, degree of hydrolysis and α are compared with those calculated.

Table XXII.

$c_d = 0{\cdot}0196$, $k_d = 6{\cdot}2 . 10^{-4}$, $c_l = 0{\cdot}0202$, $k_l = 5{\cdot}8 . 10^{-4}$, $e = 0{\cdot}0712$.

Time min.	$c_d - x_d$	x_d	$c_l - x_l$	x_l	$x_{tot.}$	$c - x_{tot.}$	$k . 10^4$ calc.	$k . 10^4$ f.	% calc.	% f.	α calc.	α f.
0	0·0196	—	0·0202	—	—	0·0398	—	—	—	—	−0·540	−0·540
5	0·0195	0·0001	0·0201	0·0001	0·0002	0·0396	4·2	7·3	0·5	0·9	−0·535	−0·530
10	0·0193	0·0003	0·0199	0·0003	0·0006	0·0392	6·6	9·4	1·5	2·2	−0·520	−0·515
20	0·0190	0·0006	0·0197	0·0005	0·0011	0·0387	6·1	7·6	2·8	3·4	−0·505	−0·500
40	0·0185	0·0011	0·0191	0·0011	0·0022	0·0376	6·2	6·7	5·5	6·0	−0·475	−0·470
60	0·0180	0·0016	0·0186	0·0016	0·0032	0·0366	6·1	6·5	8·0	8·6	−0·445	−0·440
80	0·0175	0·0021	0·0182	0·0020	0·0041	0·0357	5·9	5·9	10·3	10·3	−0·420	−0·420
110	0·0168	0·0028	0·0174	0·0028	0·0056	0·0342	6·0	6·8	14·1	15·9	−0·375	−0·355
150	0·0158	0·0038	0·0165	0·0037	0·0075	0·0323	6·0	6·1	18·8	18·9	−0·320	−0·320

Table XXIII.

$c_d = 0{\cdot}0196$, $k_d = 12{\cdot}4 . 10^{-4}$, $c_l = 0{\cdot}0202$, $k_l = 11{\cdot}5 . 10^{-4}$, $e = 0{\cdot}1424$.

Time min.	$c_d - x_d$	x_d	$c_l - x_l$	x_l	x_{tot}	$c - x_{tot.}$	$k . 10^4$ calc.	$k . 10^4$ f.	% calc.	% f.	α calc.	α f.
0	0·0196	—	0·0202	—	—	0·0398	—	—	—	—	−0·540	−0·540
5	0·0193	0·0003	0·0199	0·0003	0·0006	0·0392	13·2	18·9	1·5	2·2	−0·520	−0·515
10	0·0190	0·0006	0·0197	0·0005	0·0011	0·0387	12·2	21·0	2·8	4·7	−0·505	−0·485
20	0·0185	0·0011	0·0192	0·0010	0·0021	0·0377	11·8	16·5	5·3	7·3	−0·480	−0·455
40	0·0175	0·0021	0·0182	0·0020	0·0041	0·0357	11·8	13·9	10·3	12·0	−0·420	−0·400
60	0·0165	0·0031	0·0172	0·0030	0·0061	0·0337	12·0	12·9	15·3	16·3	−0·360	−0·350
80	0·0156	0·0040	0·0163	0·0039	0·0079	0·0319	12·0	13·7	19·8	22·3	−0·310	−0·280
110	0·0143	0·0053	0·0151	0·0051	0·0104	0·0294	12·0	12·7	26·1	27·5	−0·235	−0·220
150	0·0128	0·0068	0·0136	0·0066	0·0134	0·0264	11·9	12·2	33·7	34·3	−0·145	−0·145

Table XXIV.

$c_d = 0{\cdot}0196$, $k_d = 18{\cdot}6 . 10^{-4}$, $c_l = 0{\cdot}0202$, $k_l = 17{\cdot}3 . 10^{-4}$, $e = 0{\cdot}2136$.

Time min.	$c_d - x_d$	x_d	$c_l - x_l$	x_l	$x_{tot.}$	$c - x_{tot.}$	$k . 10^4$ calc.	$k . 10^4$ f.	% calc.	% f.	α calc.	α f.
0	0·0196	—	0·0202	—	—	0·0396	—	—	—	—	−0·540	−0·540
5	0·0192	0·0004	0·0198	0·0004	0·0008	0·0390	17·6	42·0	2·0	4·7	−0·515	−0·485
10	0·0188	0·0008	0·0194	0·0008	0·0016	0·0382	17·8	30·9	4·0	6·9	−0·495	−0·460
20	0·0180	0·0016	0·0187	0·0015	0·0031	0·0367	17·6	24·7	7·8	10·7	−0·450	−0·415
40	0·0165	0·0031	0·0172	0·0030	0·0061	0·0337	18·1	21·6	15·3	18·0	−0·360	−0·330
60	0·0152	0·0044	0·0159	0·0043	0·0087	0·0311	17·9	19·9	21·9	24·0	−0·285	−0·260
80	0·0139	0·0057	0·0147	0·0055	0·0112	0·0286	17·9	19·7	28·1	30·5	−0·210	−0·185
110	0·0122	0·0074	0·0130	0·0072	0·0146	0·0252	18·0	18·2	36·7	36·9	−0·110	−0·110
150	0·0103	0·0093	0·0111	0·0091	0·0184	0·0214	18·0	18·5	46·2	47·2	−0·000	+0·010

Here the agreement is not quite as good as in the case of the 1-methylpentyl-glucoside. The initial values differ especially. It may be that the value for e in these experiments is a little too low; if this is the case the deviations between calculated and found values will certainly be maximum in the experiment with highest emulsin concentration.

(*d*) *Determination of the equilibrium-point.* In this experiment the hydrolysis was followed during 720 minutes and an additional reading was taken after 1470 minutes. Table XXV gives the result, which is that the equilibrium-point is at some 96 % hydrolysis.

Table XXV.

$c_{emulsin}$ = 0·1896 g. in 50 ml., $\alpha_{emulsin}$ = −0·335°.
$c_{glucoside}$ = 0·0398 *M*, $\alpha_{beg.}$ = −0·540°, α_{end} = +0·625°.

Time min.	α	$\alpha_{gluc.}$	x	$c-x$	$k.10^4$	%
0·0	−0·875	−0·540	—	1·165	—	—
1·7	−0·865	−0·530	0·010	1·155	22·1	0·9
10·0	−0·815	−0·480	0·060	1·105	23·0	5·2
30·0	−0·740	−0·405	0·135	1·030	17·8	11·6
60·0	−0·640	−0·305	0·235	0·930	16·3	20·2
90·0	−0·530	−0·195	0·345	0·820	16·9	29·6
120·0	−0·450	−0·115	0·425	0·740	16·4	36·5
150·0	−0·375	−0·040	0·500	0·665	16·2	42·9
180·0	−0·305	+0·030	0·570	0·595	16·2	48·9
210·0	−0·240	+0·095	0·635	0·530	16·3	54·5
240·0	−0·190	+0·145	0·685	0·480	16·0	58·8
270·0	−0·140	+0·195	0·735	0·430	16·0	63·1
300·0	−0·090	+0·245	0·785	0·380	16·2	67·4
360·0	−0·010	+0·325	0·865	0·300	16·4	74·2
420·0	+0·060	+0·395	0·935	0·230	16·7	80·1
480·0	+0·120	+0·455	0·995	0·170	17·4	85·3
540·0	+0·165	+0·500	1·040	0·125	17·9	89·2
720·0	+0·220	+0·555	1·095	0·070	16·9	94·0
1470·0	+0·240	+0·575	1·115	0·050	—	95·7

Table XXVI gives a comparison between the calculated and the experimentally found values of k, degree of hydrolysis and α. The correspondence is here somewhat better than in Tables XXII–XXIV; this may be taken as a confirmation of the assumption that it is the value of e in the former experiments which is incorrect.

Table XXVI.

c_d = 0·0196, k_d = 16·5 . 10^{-4}, c_l = 0·0202, k_l = 15·3 . 10^{-4}, e = 0·1896.

Time min.	c_d-x_d	x_d	c_l-x_l	x_l	$x_{tot.}$	$c-x_{tot.}$	$k.10^4$ calc.	$k.10^4$ f.	% calc.	% f.	α calc.	α f.
0	0·0196	—	0·0202	—	—	0·0398	—	—	—	—	−0·540	−0·540
10	0·0189	0·0007	0·0195	0·0007	0·0014	0·0384	15·6	23·0	3·5	5·2	−0·500	−0·480
30	0·0175	0·0021	0·0182	0·0020	0·0041	0·0357	15·7	17·8	10·3	11·6	−0·420	−0·405
60	0·0156	0·0040	0·0164	0·0038	0·0078	0·0320	15·7	16·3	19·5	20·2	−0·315	−0·305
90	0·0139	0·0057	0·0147	0·0055	0·0112	0·0286	15·9	16·9	28·1	29·6	−0·215	−0·195
120	0·0124	0·0072	0·0132	0·0070	0·0142	0·0256	16·0	16·4	35·7	36·5	−0·125	−0·115
150	0·0111	0·0085	0·0119	0·0083	0·0168	0·0230	15·9	16·2	42·2	42·9	−0·050	−0·040
180	0·0099	0·0097	0·0107	0·0095	0·0192	0·0206	15·9	16·2	48·2	48·9	+0·020	+0·030
210	0·0088	0·0108	0·0096	0·0106	0·0214	0·0184	16·0	16·3	53·8	54·5	+0·085	+0·095
240	0·0079	0·0117	0·0087	0·0115	0·0232	0·0166	15·8	16·0	58·3	58·8	+0·140	+0·145
270	0·0070	0·0126	0·0078	0·0124	0·0250	0·0148	15·9	16·0	62·8	63·1	+0·190	+0·195
300	0·0063	0·0133	0·0070	0·0132	0·0265	0·0133	15·9	16·2	66·6	67·4	+0·235	+0·245
360	0·0050	0·0146	0·0057	0·0145	0·0291	0·0107	15·8	16·4	73·1	74·2	+0·310	+0·325
420	0·0040	0·0156	0·0046	0·0156	0·0312	0·0086	15·8	16·7	78·4	80·1	+0·375	+0·395
480	0·0032	0·0164	0·0037	0·0165	0·0329	0·0069	15·9	17·4	82·7	85·3	+0·425	+0·455
540	0·0025	0·0171	0·0030	0·0172	0·0343	0·0055	15·9	17·9	86·2	89·2	+0·465	+0·500
720	0·0013	0·0183	0·0016	0·0186	0·0369	0·0029	15·8	16·9	92·7	94·0	+0·540	+0·555

The figures which give a comparison of the rates of hydrolysis of the different glucosides are the values of k/e (sal. f.), taken for a constant glucoside concentration. In Table XXVII these figures are given for the glucosides here examined together with values for methylglucoside, calculated by extrapolation from the

Table XXVII.

Glucoside	Conc.	k/e (sal. f.)	$v_{rel.}$
Methyl	0·04 *M*	0·0206	1·0
1-Methylpentyl	,,	1·29	63·0
3-Methylpentyl	,,	0·186	9·0
Methyl	0·02 *M*	0·0211	1·0
d-1-Methylpentyl	,,	1·89	89·0
l-1-Methylpentyl	,,	0·548	26·0
d-3-Methylpentyl	,,	0·193	9·1
l-3-Methylpentyl	,,	0·180	8·5

experiments of Josephson [1925, p. 59]. The relative velocities are also given in the table, the velocity of hydrolysis of methylglucoside being taken as unity.

It remains to be seen if there is a proportionality between the affinity constants of the different glucosides and their rates of hydrolysis.

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CCXXXII. STUDIES IN THE METABOLISM OF THE STRICT ANAEROBES (GENUS *CLOSTRIDIUM*).

I. THE CHEMICAL REACTIONS BY WHICH *CL. SPOROGENES* OBTAINS ITS ENERGY.

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Our knowledge of the metabolism of the bacteria of the genus *Clostridium* (the strict anaerobes) is at present very scanty. It has been recently reviewed [Topley and Wilson, 1929; McLeod, 1930; Stephenson, 1930], so only a brief summary will be given here, including facts discovered since the above reviews were published. Special reference will be made to *Cl. sporogenes*, which has been used by many workers as a type species, and of which our knowledge is the fullest. The present position is as follows.

(1) These bacteria cannot grow in media containing an appreciable concentration of free oxygen. The degree of tolerance to low concentrations of oxygen varies from species to species. With regard to the reason for this lack of tolerance of oxygen no final decision has been reached. McLeod and Gordon [see McLeod, 1930] claim that it is due to production of hydrogen peroxide, while Quastel and Stephenson [1926] believe that oxygen acts by preventing the culture from reaching a sufficiently low oxidation-reduction potential.

(2) They are unable to grow except in media containing protein or amino-acids. Carbohydrates, if present in addition, are fermented, but are neither sufficient nor essential for growth.

(3) A compound containing the —SH group, or one from which the bacteria can produce an —SH group, is essential for growth (Quastel and Stephenson [1926] for *Cl. sporogenes*, and Burrows [1933] for *Cl. botulinum*).

(4) Tryptophan is an "essential" amino-acid for *Cl. sporogenes* [Fildes and Knight, 1933]. With other species opinions differ; for instance, with *Cl. botulinum* Fildes and Knight say that tryptophan is "essential," Burrows [1933] that it is not. The word "essential" is here used in the special sense that the bacteria require it because they are unable to synthesise it.

(5) In the case of *Cl. sporogenes* growth depends also on the presence in the medium of a trace of a vitamin-like substance [Knight and Fildes, 1933].

(6) Burrows [1933] showed that *Cl. botulinum* can grow on a synthetic medium containing no amino-acids except glycine, alanine, leucine, proline, lysine and cysteine.

(7) Wolf and Harris [1917; 1918–19] analysed the products of the action of *Cl. sporogenes* on various natural media, but could deduce little as to the reactions by which the products had been formed.

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It is evident that the quantitatively most important part of the chemical action of bacteria on the medium during growth must be the chemical reactions by which they obtain their energy. As the above summary shows, we know nothing so far of this aspect of the metabolism of the strict anaerobes, and it forms the subject of the present communication.

THE CHEMICAL REACTIONS BY WHICH *CL. SPOROGENES* OBTAINS ITS ENERGY.

The simplest medium on which *Cl. sporogenes* will grow consists of an acid hydrolysate of gelatin with the addition of traces of cysteine (or thiolacetic acid), tryptophan and vitamin [Knight and Fildes, 1933]. This medium contains practically nothing but amino-acids, so the source of energy must lie in anaerobic reactions involving only amino-acids. It is difficult to picture any reaction by which a single amino-acid molecule could break down anaerobically to yield energy, so the most likely type of reaction is one between two molecules of amino-acids, probably involving oxidation of one molecule and reduction of the other, as in the hypothetical equation

$$R^1.CHNH_2.COOH + R^2.CHNH_2.COOH + H_2O \rightarrow R^1.CO.COOH + R^2.CH_2.COOH + 2NH_3.$$

R^1 and R^2 may theoretically be the same group or two different groups.

A reaction of this type would be similar to certain anaerobic energy-yielding reactions of *Bact. coli*, *e.g.* sodium lactate and sodium fumarate reacting to give sodium pyruvate and sodium succinate [Quastel *et al.*, 1925]. If such a reaction between amino-acids were catalysed by *Cl. sporogenes*, it would be expected that some amino-acids would react as hydrogen donators (*i.e.* would reduce methylene blue in the presence of the bacteria) while others would act as hydrogen acceptors (*i.e.* would oxidise leucomethylene blue). These theoretical considerations have been tested by experiment, and found to provide a satisfactory explanation of the method by which *Cl. sporogenes* obtains its energy.

EXPERIMENTAL.

Preparation of bacterial suspensions.

All the experiments described in this paper were done with washed suspensions of *Cl. sporogenes*. The culture was obtained from the National Collection of Type Cultures (No. 533, *B. sporogenes* Bellette), and was maintained on a meat medium. To prepare the washed suspensions, 800 ml. of tryptic digest of caseinogen in a one litre flask were inoculated directly with a loopful of the meat culture, and incubated for 40–45 hours in a McIntosh and Fildes anaerobic jar. The culture was then centrifuged, and the bacteria were washed twice by centrifuging with Ringer's solution and finally suspended in 10–20 ml. of Ringer's solution. The enzymic activity of such suspensions was very unstable, and experiments had to be carried out within a few hours of the washing of the bacteria; within 24 hours the activity had completely disappeared, even if the suspension were kept anaerobically in the ice-chest. Microscopic examination of many of the suspensions prepared in this way showed that they almost always contained nothing but vegetative cells, though rarely a few spores were visible.

Detection of hydrogen donators.

The usual method was employed. 1 ml. of phosphate buffer at p_H 7·5, 1 ml. of methylene blue or brilliant cresyl blue $M/2000$, 1 ml. of water and 1 ml. of the substances under investigation in neutralised $M/10$ solutions were added to a series of Thunberg tubes. After the addition to each in turn of 1 ml. of a

suitably diluted bacterial suspension, the tubes were evacuated and incubated in a water-bath at 40°, and the rate of reduction of the dye was observed. The averages of the results of a large number of such experiments are given in Table I. It will be noted first that those substances which are the most active

Table I. *Relative velocities of oxidation by dyes of various substrates. Velocity with alanine is taken as standard (=100).*

Substrate	Rate of oxidation	Substrate	Rate of oxidation
Sodium formate	0	*l*-Leucine	100
Sodium acetate	0	*l*-Phenylalanine	10
Sodium propionate	0	*l*-Aspartic acid	5
Sodium lactate	0	*d*-Glutamic acid	2
Sodium succinate	0	*d*-Arginine	0
Sodium pyruvate	40	*d*-Lysine	0
Glucose	0	*l*-Histidine	<2
		l-Proline	0
Glycine	0	*l*-Hydroxyproline	0
d-Alanine	100	*dl*-Serine	0
l-Alanine	0	*l*-Tyrosine	<2
d-Valine	60	*l*-Tryptophan	<2

hydrogen donators in the case of facultative anaerobes and aerobes (*e.g.* glucose, formate, lactate and succinate) were not oxidised at all by *Cl. sporogenes*. On the other hand, among the amino-acids were several very active hydrogen donators, especially the simple aliphatic monoamino-acids, *d*-alanine, *d*-valine and *l*-leucine. The only compound among those tried, apart from the amino-acids, which could be oxidised at all was sodium pyruvate.

The oxidation of alanine.

Before proceeding further, a few experiments on the reaction between alanine and methylene blue or cresyl blue must be described.

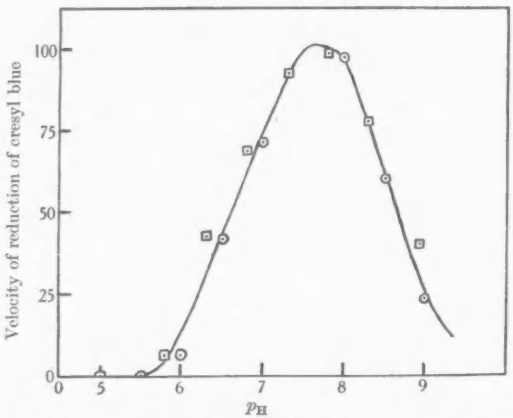

Fig. 1. Relation between activity of alanine dehydrogenase and p_H.

(*a*) *The relation between rate of oxidation and* p_H. A series of Thunberg tubes containing 1 ml. *M*/10 *d*-alanine, 1 ml. *M*/2000 cresyl blue, 1 ml. water, 1 ml. suspension of bacteria and 1 ml. of buffer (phthalate, phosphate or borate

according to the p_H) at different p_H values was incubated at 40° and the reduction time of the dye noted. The optimum p_H was found to lie at about 7·6 (see Fig. 1). A similar result was obtained with *l*-leucine.

(*b*) *The relation between rate of oxidation and concentration of alanine.* A similar series of tubes, all at p_H 7·5, with different concentrations of *d*-alanine was incubated at 40° and the reduction time of the dye observed. It was found that the affinity of alanine dehydrogenase for *d*-alanine was rather low ($K = 10^{-2·5}$ *M*), so that maximum velocity is not obtained unless the concentration of alanine is about *M*/30.

(*c*) *Stereochemical specificity.* *l*-Alanine hydrochloride was prepared from the commercial *dl*-product by the method of Fischer [1899]. The sample of *l*-alanine prepared was too small to permit of a measurement of its rotation, which is very low, but the rotation of the benzoyl-*l*-alanine was measured before hydrolysis, and found to be of the right order (found $[\alpha]_{5461}^{16·5°} = -36°$; Fischer [1899] gives $[\alpha]_D^{20°} = -37·4°$). As the result was a complete negative, exact proof of the optical purity of the specimen was unnecessary. A neutralised *M*/10 solution was added to a Thunberg tube in the usual way and was found not to reduce methylene blue. To eliminate the unlikely possibility of some toxic material being present in the preparation, a control experiment was carried out as below:

	Reduction time (min.)
1 ml. *d*-alanine *M*/10	7½
1 ml. *l*-alanine hydrochloride *M*/10	<120
1 ml. *d*-alanine + 1 ml. *l*-alanine hydrochloride	9½

Specimens of the enantiomorphs of other natural amino-acids have not yet been obtained, but it seems likely that only the natural isomerides are oxidised.

(*d*) *The course and extent of oxidation of alanine.* The usual method of measuring the extent of oxidation of a substrate, *viz.* manometric measurement of oxygen uptake, was not available in this case. Even in the presence of a dye as hydrogen carrier no oxygen was used. This might be due to peroxide formation, but addition of purified catalase to the solutions in the manometer vessel did not enable the oxidation to proceed. The only alternative method of following the oxidation was to allow the bacteria to reduce a strong solution of an indicator, and for this purpose brilliant cresyl blue was chosen in preference to methylene blue for three reasons: (*a*) it is more quickly reduced, (*b*) it is less toxic, (*c*) its leuco-form is more soluble in water. The potential of cresyl blue (+0·043 v.) is slightly higher than that of methylene blue (+0·011 v.).

The method employed was as follows. In a 100 ml. Büchner flask were placed 5 ml. *M*/5 phosphate buffer p_H 7·5, 10 ml. of a thick suspension of *Cl. sporogenes* and a known amount (usually of the order of 0·005 millimol) of *d*-alanine dissolved in 5 ml. of water. The flask was fitted with a rubber stopper carrying a 10 ml. tapped burette with a fine jet. The flask was now thoroughly evacuated, sealed and immersed in a water-bath at 40°. The burette was filled with a freshly de-aerated *M*/200 solution of cresyl blue, and after an interval of 5 minutes to allow the solutions to come to the temperature of the bath, this solution was run in drop by drop, a further drop being added as soon as the previous one was reduced. The time was noted after the reduction of each 0·2 ml. With every such experiment a control was carried out, differing only in the omission of the alanine.

In Fig. 2 are shown graphically the results of one experiment. It will be noted that the reduction in the control is very large, so that the end-point of the oxidation of the alanine is rather vague. Fig. 3 gives the results of a series

of experiments, all corrected for the blank. These curves show that for each molecule of alanine two molecules of the dye are reduced rapidly. In some

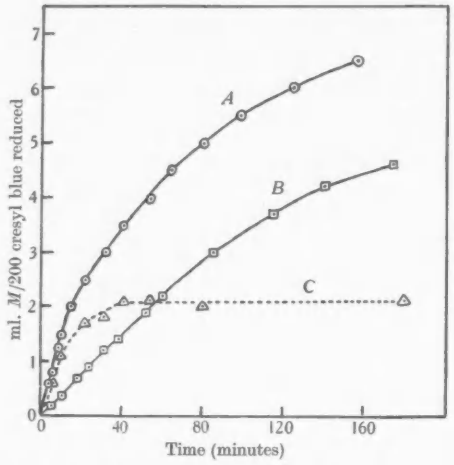

Fig. 2. Course of oxidation of *d*-alanine by cresyl blue. Curve *A*, 1 ml. *M*/200 alanine; curve *B*, no alanine; curve *C*, difference between *A* and *B*.

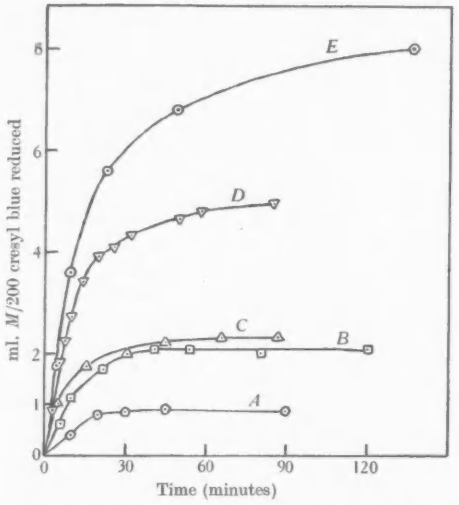

Fig. 3. Course of oxidation of *d*-alanine by cresyl blue. *A*, 0·4 ml., *B* and *C*, 1·0 ml., *D*, 2·0 ml. and *E* 4·0 ml. of *M*/200 *d*-alanine, all corrected for blank.

cases there appears to be a further very slow oxidation, but on account of the large correction that has to be made for the blank it is very doubtful whether this has any significance.

It was found impossible to reduce this "blank" reduction by further washing of the bacteria, or to remove it by chemical treatment of the suspensions. For instance, toluene treatment, which is so useful with *Bact. coli*, completely destroys the enzymes of *Cl. sporogenes*. In these particular experiments the blank is, of course, greatly exaggerated, as the substrate concentration is so small ($M/4000$). At higher substrate concentrations the blank would be proportionately much smaller both in amount and in rate, but higher concentrations of alanine could not be used on account of the toxic properties of large amounts of cresyl blue.

(*e*) *The deamination of alanine during oxidation.* From experiments such as those described in the preceding paragraph it is possible to learn at what stage in the oxidation of alanine its amino-group is eliminated as ammonia. For this purpose an experiment and its control were taken down when the (corrected) amount of dye reduced was rather more than the molecular equivalent of the alanine present. The dye was removed with kieselguhr, and the ammonia estimated in aliquot portions of the filtrates. Other experiments were carried out with excess of alanine in the flask (5 ml. $M/10$). When a certain amount of the dye had been reduced, the solutions were treated as above, and the ammonia was estimated. One result from each type of experiment is given in Table II. It is

Table II.

Alanine present	Cresyl blue reduced (corrected for blank) ml. $M/100$	Ammonia found (corrected for blank) ml. $M/100$
5 ml. $M/100$	7·0	5·3
10 ml. $M/20$	3·75	3·40

clear that in the first case the whole of the alanine had been deaminated while only 40 % excess of dye had been reduced, and in the second case the ammonia corresponded to the amount of dye reduced. The alanine is therefore deaminated during the first step of the oxidation.

Detection of hydrogen acceptors.

A hydrogen acceptor is a substance which, in the presence of cells, will oxidise leuco-dyes. The easiest method of demonstrating such substances is that devised by Quastel and Whetham [1924], in which the bacteria themselves are allowed to reduce the indicator before the substance to be tested is added from a side-tube. In the present work the original U-tube of Quastel and Whetham was replaced by the Keilin modification of the Thunberg tube, which has a hollow stopper capable of holding up to 0·5 ml. of the solution of the substance under investigation.

Each tube contained 1 ml. of buffer, $M/5$ p_H 7·5, 1 ml. of a thick suspension of *Cl. sporogenes*, 1 ml. of indicator (usually $M/2000$, but sometimes less, according to the tinctorial power of the indicator), and 1 ml. of leucine $M/100$ (sometimes, owing to the great reducing blank of the thick bacterial suspensions, the leucine could be omitted). The stopper contained 0·2 ml. of a neutralised $M/2$ solution of the substance to be tested, or else the equivalent amount of the dry solid. The tubes were thoroughly evacuated, special care being taken that no dissolved air remained in the solution in the stopper, and incubated at 40° until the dye was completely reduced. At this point the solution in the stopper was tipped into the tube and the whole left for a further 2 hours to observe any reoxidation of the indicator. In the first series of experiments the traditional methylene blue was used, and no substance was found capable of

oxidising leucomethylene blue in the presence of *Cl. sporogenes*. This meant that, if hydrogen acceptors with respect to *Cl. sporogenes* exist, they must lie at a considerably more negative point on the potential scale than methylene blue, and consequently experiments were carried out with more negative dyes. It was obviously useless to employ dyes so negative that the dehydrogenases of *Cl. sporogenes* were incapable of reducing them, so a rough colorimetric determination was made of the reducing level of the alanine dehydrogenase system. It was found that all dyes down to benzyl viologen were easily reduced (r_H 3 at p_H 7·5), and a partial reduction even of methyl viologen at p_H 8·0 (r_H 1) was observed.

Another series of experiments was therefore carried out with benzyl viologen. It was now found that proline and hydroxyproline caused a rapid and complete reoxidation of this dye, while glycine caused a rapid partial reoxidation. No other substance tried had any effect, and it is noteworthy that, as in the case of hydrogen donators, substances which are active with facultative anaerobes (*i.e.* nitrate and fumarate) are inactive with *Cl. sporogenes* (see Table III).

Table III. *Hydrogen acceptors.*

Substance	Indicator	r_H of indicator	Reoxidation
Sodium nitrate	Benzyl viologen	3 (at p_H 7·5)	−
Sodium fumarate	,,	,, ,,	−
dl-Serine	Benzyl viologen	3 (at p_H 7·5)	−
l-Aspartic acid	,,	,, ,,	−
d-Glutamic acid	,,	,, ,,	−
d-Arginine	,,	,, ,,	−
d-Lysine	Neutral red	,,	−
l-Histidine	Benzyl viologen	,, (at p_H 7·5)	−
l-Tyrosine	,,	,, ,,	−
l-Proline	Benzyl viologen	3 (at p_H 7·5)	+ +
,,	Neutral red	,,	+ +
,,	Rosinduline	4·5	+ +
,,	Phenosafranine	5·5	+ +
,,	Ethyl Capri blue	11·5	−
l-Hydroxyproline	Benzyl viologen	3 (at p_H 7·5)	+ +
,,	Neutral red	,,	+ +
Glycine	Methyl viologen	1 (at p_H 8·0)	+ +
,,	Benzyl viologen	3 (at p_H 7·5)	+
,,	Phenosafranine	5·5	−

+ + indicates complete reoxidation. + indicates partial reoxidation.

A few further experiments were done to determine the oxidising level of glycine and proline (Table III). Glycine oxidised methyl viologen completely, benzyl viologen partially, and phenosafranine not at all, so it lies somewhere near the range of benzyl viologen (r_H 3 at p_H 7·5). Proline reoxidised all the leuco-dyes up to phenosafranine (−0·25 v.), but failed to oxidise leuco-Capri blue (−0·08 v.), so it lies somewhere between these two values. No suitable indicator between these exists, since *Cl. sporogenes* is unable to reduce Nile blue (−0·125 v.) or cresyl violet (−0·15 v.).

The reduction of glycine and proline.

The study of the reduction of hydrogen acceptors by a method analogous to that used in following the oxidation of alanine, *i.e.* by letting the bacteria oxidise a strong standard solution of a leuco-dye, presents great technical difficulties and has not been attempted. There are accounts in the literature however

of the reduction of these substances by mixed cultures of bacteria, which suggest the probable course of the reductions in the present experiments. Brasch [1909] showed that glycine was converted by putrefying bacteria into acetic acid and ammonia; this is indeed the only way in which glycine can be reduced, unless the biologically abnormal reduction of the carboxyl group is considered. Neuberg [1911] allowed mixed putrefying bacteria to act on proline and isolated from the products δ-aminovaleric acid and *n*-valeric acid. The first stage of the reduction in this case apparently breaks the ring, without deamination. Ackermann [1911] also obtained the same products from proline. Keil and Günther [1933], from similar experiments with hydroxyproline, isolated only δ-aminovaleric acid.

Direct reactions between hydrogen donators and hydrogen acceptors.

It has so far been demonstrated that *Cl. sporogenes* is able to activate certain amino-acids, some as hydrogen donators (*e.g.* alanine, valine and leucine) and some as hydrogen acceptors (proline, hydroxyproline and glycine). For the provision of energy for the bacteria it is obviously not sufficient that these substances should react with dyes and leuco-dyes respectively; they must be able tc react directly with one another, without the intervention of an artificial hydrogen carrier. For detecting such reactions between hydrogen-donating and hydrogen-accepting amino-acids, a useful test is provided in the fact, already demonstrated, that the amino-group of alanine is eliminated as ammonia during the first stage of oxidation. Hence if, for instance, alanine is oxidised by proline, ammonia will be liberated.

Experiments were carried out in the following way. In a Thunberg tube were placed 0·5 ml. of a *M*/10 solution of the hydrogen donator, 0·5 ml. of a *M*/10 solution of the hydrogen acceptor, 0·5 ml. of *M*/5 phosphate buffer, p_H 7·5, and 1 ml. of a suspension of *Cl. sporogenes*. Control tubes contained (*a*) hydrogen donator alone, (*b*) hydrogen acceptor alone and (*c*) neither amino-acid. The tubes were evacuated and incubated at 40° for a period of usually 2–6 hours. After incubation the free ammonia was estimated in each tube. 2 ml. of the solution were measured into 25 ml. of 50 % alcohol in a Kjeldahl flask, which was connected through a condenser to a receiver containing 5 ml. of *N*/100 sulphuric acid. The solution was made alkaline by the addition of 10 ml. of borate buffer (*M*/5, p_H 10), and raised to the boiling-point, while the ammonia was removed to the receiver by a current of ammonia-free air. The excess of acid was titrated with *N*/100 CO_2-free NaOH. A typical result with alanine and proline is shown in Table IV. This shows that only when hydrogen donator

Table IV.

	Ammonia found ml. *N*/10	Ammonia (corrected for blank) ml. *N*/10
Blank	0·07	—
0·5 ml. *M*/10 *d*-alanine	0·06	0·00
0·5 ml. *M*/10 *l*-proline	0·05	0·00
0·5 ml. alanine + 0·5 ml. proline	0·42	0·35

and hydrogen acceptor were present together was any ammonia liberated, and this is good evidence that the reaction proceeding was indeed an oxidation of alanine and reduction of proline. Further evidence on this point will be given later.

It can further be shown that any one of the hydrogen-accepting amino-acids will react in the same way with any one of the hydrogen-donating amino-acids. Experiments with *d*-alanine, *d*-valine and *l*-leucine on the one hand, and glycine, *l*-proline and *l*-hydroxyproline on the other, carried out in the way described above, gave the results shown in Table V. None of the amino-acids alone is deaminated, but any mixture of hydrogen donator and acceptor produces ammonia.

Table V. *Direct reactions between pairs of amino-acids.*

0·5 ml. of *M*/10 solution of each amino-acid was used.
The figures represent ammonia liberated (ml. *N*/10 per tube) corrected for the blank experiment.
The first figure is for the hydrogen acceptor alone, the second for the hydrogen donator alone, and the third for both together.

	Hydrogen donators		
Hydrogen acceptors	*d*-Alanine	*d*-Valine	*l*-Leucine
l-Proline	0·00	0·00	0·00
	0·00	0·00	0·02
	0·35	0·33	0·20
l-Hydroxyproline	0·00	0·00	0·00
	0·00	0·00	0·02
	0·28	0·41	0·15
Glycine	0·01	0·04	0·04
	0·00	0·00	0·01
	0·26	0·55	0·31

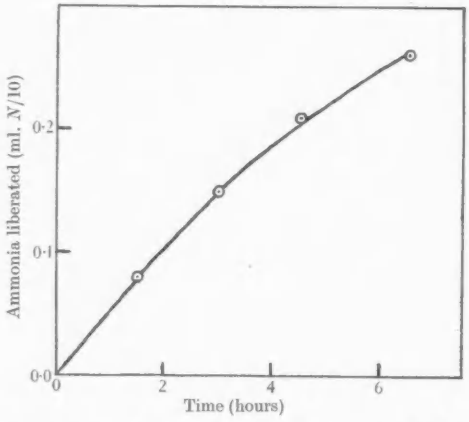

Fig. 4. Course of reaction between glycine and *d*-alanine.

(*a*) *The course of the reaction.* It is necessary to exclude the possibility that the bacteria were growing during these rather long experiments; this is, of course, unlikely as *Cl. sporogenes* has never been shown to grow on such simple mixtures, and has, even when growing on tryptic broth, a lag period of about 24 hours. A set of Thunberg tubes was prepared containing 0·5 ml. of buffer, 0·5 ml. *M*/10 *d*-alanine, 0·5 ml. *M*/10 glycine and 1 ml. of bacterial suspension, and another set with 1 ml. of water replacing the two amino-acids. These were incubated at 40°, and the ammonia liberated was estimated at various times up to 6 hours. The result is shown in Fig. 4 (ammonia liberated, corrected for control,

plotted against time), and it is clear that the reaction started with no lag period and proceeded with slowly diminishing velocity.

Another similar experiment showed that the glycine and alanine were both completely deaminated.

	Ammonia found (6½ hours) (corrected for control) ml. $N/10$
0·5 ml. $M/10$ glycine + 0·5 ml. $M/10$ alanine	0·95

It can further be shown, in the same way, that just as one molecule of alanine can reduce two of cresyl blue, so it can reduce two molecules of glycine.

	Ammonia found (22 hours) (corrected for control) ml. $N/10$
1·0 ml. $M/10$ glycine + 0·5 ml. $M/10$ alanine	1·46

(*b*) *Energy of reaction and rates of reaction.* It is clear now that *Cl. sporogenes* is able to carry out various oxidation-reduction reactions anaerobically between pairs of amino-acids. To decide whether these reactions are of quantitative significance in the metabolism of the bacteria two further points must be considered: (*a*) the amount of energy liberated by the reactions and (*b*) the rates at which they proceed, relative to the amount of cell material present. Unfortunately the data are not available for calculating the change of free energy in any of these reactions, so for the time being this point must be passed over, but experiments on the rates of the reactions show that they proceed at a speed comparable with those of other bacterial oxidations. That some energy is liberated by the reactions is of course demonstrated by the fact that they do proceed spontaneously.

The velocity of the reaction between glycine and *d*-alanine or *l*-proline and *d*-alanine was measured by estimating the rate of ammonia production as described above, and the nitrogen content of the bacterial suspension was determined by a micro-Kjeldahl method. Using the fact that 10 % of the dry weight of *Cl. sporogenes* is nitrogen, the dry weight of the bacteria used in the experiment was calculated. The results are most conveniently expressed in terms of $Q_{O_2}^{\text{alanine}}$, this being taken to mean in the present case the number of μl. of oxygen which would be used per mg. dry weight of bacteria per hour if the oxidation had been aerobic. Allowing for the fact that in the reaction between alanine and glycine only one-half of the ammonia is derived from the alanine, it was found in nine experiments that $Q_{O_2}^{\text{alanine}}$ with glycine as oxidant varied from 5 to 12. Considering the great instability of the enzymes concerned, it appears justifiable to take the highest value recorded as a minimum, and on that assumption the values given in Table VI were obtained. These may be compared with the figures of Cook and Haldane [1931] for aerobic oxidations with *Bact. coli*, *viz.* $Q_{O_2}^{\text{formate}} = 40$, $Q_{O_2}^{\text{lactate}} = 12$ *etc.*

Table VI. *Rates of oxidation in terms of dry weight of bacteria.*

	Hydrogen donators		
	d-Alanine	*d*-Valine	*l*-Leucine
Hydrogen acceptors	Q_{O_2}	Q_{O_2}	Q_{O_2}
Glycine	12	12	8
l-Proline	50	—	30
l-Hydroxyproline	40	—	—

(c) *Reactions between other amino-acids.* The fact that glycine when reduced, and alanine when oxidised, liberate ammonia, can be used to confirm the identity of hydrogen donators and hydrogen acceptors. The indicator experiments already described, though useful as a starting-point, are not physiological, and cases are known in which substances which do not react with indicators do react with natural substrates, *e.g. Bact. coli* oxidises acetate rapidly by means of oxygen but not at all by means of methylene blue. A large number of amino-acids were therefore tested as hydrogen donators by estimation of the ammonia produced when they were incubated anaerobically with glycine and a suspension of *Cl. sporogenes*, and as hydrogen acceptors by estimation of the ammonia production when they were incubated with *d*-alanine. The experiments were carried out as before, *i.e.* 0·5 ml. of *M*/10 solution of each amino-acid, 0·5 ml. of buffer at p_H 7·5 and 1 ml. of suspension of bacteria were incubated anaerobically in Thunberg tubes at 40°. After 2–5 hours' incubation, the ammonia was estimated in 2 ml. of the solution. The usual controls with neither amino-acid and with each separately were performed.

The results of incubation of various amino-acids with glycine are shown in Table VII. It will be seen that there is a fairly close agreement between the

Table VII. *Hydrogen donators.*

	By reduction of indicator	By ammonia from glycine		By reduction of indicator	By ammonia from glycine
Glycine	0	0	*d*-Arginine	0	0
d-Alanine	100	100	*d*-Lysine	0	0
d-Valine	60	100	*l*-Histidine	<2	55
l-Leucine	100	70	*l*-Proline	0	0
l-Phenylalanine	10	30	*l*-Hydroxyproline	0	0
l-Aspartic acid	5	25	*l*-Tryptophan	<2	10
d-Glutamic acid	2	15			

indicator method and the glycine method, but that some amino-acids, notably glutamic acid and histidine, react relatively more quickly with glycine than with the indicator.

The tests for hydrogen acceptors by incubation with alanine gave the results shown in Table VIII. The agreement is complete, and only glycine, proline and hydroxyproline can oxidise either alanine or leuco-dyes in the presence of *Cl. sporogenes*.

Table VIII. *Hydrogen acceptors.*

	By oxidation of leuco-indicators	By ammonia from alanine		By oxidation of leuco-indicators	By ammonia from alanine
Glycine	+	+	*d*-Arginine	–	–
d-Alanine	–	–	*d*-Lysine	–	–
d-Valine	–	0	*l*-Histidine	–	–
l-Leucine	–	–	*l*-Proline	+	+
l-Aspartic acid	–	–	*l*-Hydroxyproline	+	+
d-Glutamic acid	–	–	*l*-Tryptophan	0	–

0 = not tested.

Serine and tyrosine.

It was found in the course of the above experiments that serine and tyrosine behaved exceptionally in liberating ammonia when incubated alone with *Cl. sporogenes*. Neither amino-acid reacted with dyes either as a hydrogen

donator or as a hydrogen acceptor, except for a very slight hydrogen-donating activity of tyrosine. The production of ammonia from serine was demonstrated by experiments similar to those already described, but, since only *dl*-serine was available, 0·5 ml. of an *M*/5 solution of this compound was used. The following is a typical experimental result:

	Ammonia production in 7 hours ml. *N*/10 per tube
0·5 ml. *M*/10 glycine	0·04
0·5 ml. *M*/10 *d*-alanine	0·00
0·5 ml. *M*/5 *dl*-serine	0·35
0·5 ml. glycine + 0·5 ml. serine	0·53
0·5 ml. alanine + 0·5 ml. serine	0·35

Thus serine, apart from yielding ammonia by itself, donates hydrogen to glycine to give extra ammonia, but does not accept hydrogen from alanine. The rate of production of ammonia from serine is about the same as that from a mixture of glycine and alanine. Tyrosine gives ammonia at about one-fifth of this rate, and does not yield extra ammonia with either glycine or alanine. The slow rate may be caused by the small solubility of tyrosine in water (this amino-acid was added as a *M*/10 suspension).

The chemistry of the reactions.

Preliminary results show that the complete reaction between *d*-alanine and glycine is

$$CH_3.CHNH_2.COOH + 2CH_2NH_2.COOH + 2H_2O \rightarrow 3CH_3COOH + 3NH_3 + CO_2.$$

The evidence for this will be presented in a later paper. Work is proceeding on the chemistry of the oxidation of *l*-leucine and the reduction of *l*-proline, and also on the application of these results to growth experiments with *Cl. sporogenes*.

Discussion.

The experiments described in this paper show that washed cells of *Cl. sporogenes* are able to catalyse a hitherto unknown type of chemical reaction, *viz.* the oxidation and reduction of certain pairs of amino-acids. Whether these reactions play an important quantitative part in the provision of energy for the growth of the bacteria on protein media has not yet been determined experimentally, but there can be little doubt that they do. The results of Burrows [1933] are in support of this view. He found that *Cl. botulinum* grew well on a mixture of amino-acids containing only glycine, *d*-alanine, *l*-leucine, *l*-proline, *d*-lysine, *l*-cysteine and *l*-tryptophan. It is significant that, excluding the two "essential" amino-acids cysteine and tryptophan, two of the remaining five are hydrogen donators (alanine and leucine) and two hydrogen acceptors (glycine and proline). Burrows [1932; 1933] interpreted his results as showing that all these amino-acids were "essential," *i.e.* the bacteria needed their presence in the medium because they were unable to synthesise them, but this seems improbable, especially in the case of such simple molecules as glycine and alanine. It is far more likely that they are needed to provide energy by their chemical interaction.

It was stated in the introduction to this paper that "it is difficult to picture any reaction by which a single amino-acid molecule could break down anaerobically to yield energy." It now seems possible that the case of serine is an example of this type of reaction, though it is equally possible that this is an example of a reaction between two molecules of the same amino-acid. It is

useless to speculate on the chemistry of the breakdown of serine, and the author hopes to study the matter experimentally.

Bessey and King [1934] have recently described experiments in which washed suspensions of *Cl. sporogenes* were allowed to act aerobically on various amino-acids. They found that the amino-acids were deaminated, some completely. These results are not comparable with those described in the present paper, since, apart from the aerobic conditions, the reactions were very slow (alanine deaminated in 5 days, arginine in 3 days, glutamic acid in 7 days, *etc.*), and there was in every case a considerable lag period before the reaction started. The significance of such deaminations is not yet clear.

Two interesting points should be mentioned in connection with the oxidation of alanine by *Cl. sporogenes*. Krebs [1933] in studying the aerobic oxidation of amino-acids by mammalian tissue slices found that though the natural optical isomerides were oxidised their enantiomorphs were oxidised with much greater velocity by kidney slices. This is the opposite of the case with *Cl. sporogenes*, where the non-natural isomeride is not oxidised at all. Yeast [Ehrlich, 1906] and moulds [Schultze and Bosshard, 1906] also attack only the natural isomerides. Wurmser and Mayer-Reich [1933] calculated the free energy change of the reaction

$$CH_3.CO.COO^- + NH_4^+ + 2H \rightarrow CH_3.CHNH_2.COOH + H_2O$$

and showed that an alanine dehydrogenase system should reduce indicators down to about $-0{\cdot}06$ v. (r_H 12) but no further. The alanine dehydrogenase of *Cl. sporogenes* reduces dyes down to $-0{\cdot}35$ v. (r_H 2), so the only conclusion is that pyruvic acid is not in this case the oxidation product of alanine. Experimental evidence on this point will be given in a later paper.

Summary.

Washed suspensions of *Cl. sporogenes* activate certain amino-acids as hydrogen donators (especially alanine, valine and leucine) and others as hydrogen acceptors (glycine, proline and hydroxyproline).

One molecule of alanine reduces two molecules of cresyl blue; the amino-acid is deaminated during the first step of the oxidation.

Direct reactions occur between the hydrogen-donating and hydrogen-accepting amino-acids, any one of one group reacting with any one of the other.

The rates of these reactions are of the same order as those of aerobic oxidations with other bacteria, *e.g.* $Q_{O_2}^{\text{alanine + proline}} = 50$.

The probable significance of these reactions in the metabolism of the bacteria is discussed.

The author wishes to thank Sir F. G. Hopkins and Miss M. Stephenson for their interest and help in this work.

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CCXXXIII. DIFFUSION EQUILIBRIA FOR THE ISOLATED FROG'S KIDNEY.

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The isolated sartorius of the frog has been selected as particularly suitable for the study of the phenomena of muscular contraction owing to the quick diffusion of oxygen and metabolites across its substance, and the length of time it will therefore retain its normal activity. Here for similar reasons we shall consider the diffusion equilibria of the isolated frog's kidney (which in breadth and thickness resembles the sartorius muscle) with respect to substances introduced into a surrounding Locke's fluid. In the active isolated kidney of the frog without any flow of urine diffusion equilibria of substances in an external Locke's fluid are comparatively quickly attained. They are characteristic for the kidney and must be related to its special activity. The comparison is made with similar equilibria in kidneys under the influence of cyanide and hence inactive, and also with the sartorius muscle and other tissues.

The equilibrium concentrations of the inactive kidney will in general be lower than that of the surrounding fluid, since even with the freest permeability the amount of water available for solution is only about 80 % of the volume of the tissue. Where a substance under investigation is one which is normally concentrated in the urine we may expect, from the theory of active secretion, that its equilibrium concentration will exceed that of the inactive kidney.

There existed the possibility that the urine within the tubule of such kidneys would be so small in quantity that the difference between activity and non-activity in secretion could not be satisfactorily demonstrated in such a manner. The results have however shown unexpectedly large differences. For urea, phenol red and bromophenol blue (three typical "secreted" substances) not only do the equilibrium concentrations always exceed those for the inactive kidney but in many instances considerably exceed that of the substance in the surrounding Locke's fluid.

Where the substance under consideration, judging from the analysis of the frog's urine and blood, is diluted in the urine (*i.e.* is a re-absorbed substance), the equilibrium concentration in the active kidney may be expected to be less than in the inactive kidney. From a consideration of the behaviour of the mammalian kidney it was surprising to find that sodium sulphate was in general for the frog (*Rana temporaria*) a strongly re-absorbed substance, in the sense that the concentration of sulphate in the urine of the normal frog was found, in about 80–90 % of cases, to be only one-third or so of that of the plasma. In about 10–20 % of urines examined the relationship was the reverse, the concentration in the urine being about three to eight times that in the plasma. In a similar way diffusion equilibria for sulphate in the active isolated kidney

were in the great majority of cases (85 %) markedly less than those of the inactive kidney and for a small group markedly above.

The unexpected relations of sodium sulphate in the frog's kidney and the verification of certain principles believed to underlie renal secretion, already propounded elsewhere [Conway and Kane, 1929], led to a fuller investigation of this substance and to the collection of a large number of data on the sulphate equilibria of the kidney supplemented by observations on other tissues. These will be presented in this communication.

Methods.

Analytical procedure. Different methods for the analysis of minute quantities of sulphate have been recently described. Lorant [1929] converts the sulphate into hydrogen sulphide and estimates it as such; Van Slyke *et al.* [1927] determine it ultimately as a volume of nitrogen which is in relation to the original sulphate, and Hubbard [1927] precipitates as benzidine sulphate which is determined colorimetrically after oxidation with hydrogen peroxide and ferric chloride. The method selected at first was Wakefield's modification of Hubbard's method [1929]; later the benzidine sulphate precipitates were titrated in the manner described by Cope [1931]. Both colorimetric and titrimetric methods were found to give essentially similar results; the results to be described were however obtained (unless otherwise stated) by the titration method. The microburette used for the titration was not that of Rehberg [1925] but one described in a previous communication [Conway, 1934]. It is as accurate as the Rehberg burette and may be easily constructed in the laboratory. For the present determinations it contained 0·005N CO_2-free alkali. The procedure adopted in analysing the kidneys for sulphate was as follows. The kidneys (usually two in number) were removed. They were then transferred to a weighed watch glass and rapidly weighed. After weighing they were transferred to a clean, dry, agate mortar with 1 ml. of distilled water and a little quartz sand (Merck's purest) and ground to a fine suspension in 2 minutes. (The quartz sand is in fact scarcely necessary as the kidneys are rapidly disintegrated in distilled water.) 1 ml. of 13·5 % trichloroacetic acid was now added and stirred with the mixture for another minute, and 2 ml. of distilled water were added and mixed. The fluid was subsequently transferred to a 15 ml. centrifuge-tube and centrifuged for about 5 minutes to clear the suspension. 3 ml. of the clear fluid were then taken, 10 ml. of 2 % benzidine in pure acetone added and the procedure was subsequently the same as that described by Cope. The final titration was carried out after adding 1 ml. of a weak aqueous solution of phenol red having the end-point colour. We found the phenol red more sensitive than phenolphthalein. Suitable blanks and controls were included in each series analysed. Since a knowledge of the sensitivity of the method is of importance in a consideration of the results, the following account may be given of the estimation of the sulphate in 0·1 ml. of a sodium sulphate solution containing 10 mg. S per 100 ml. This was delivered into centrifuge-tubes with the addition of water and trichloroacetic acid to bring to the usual trichloroacetic acid strength, the volume being 3 ml. before adding the benzidine reagent. Table I gives an account of the data obtained in the first five of a series of 24 such analyses. The standard deviation of the single determination was $0{\cdot}6 \times 10^{-3}$ mg. S, the 24 analyses being contained within the range $-1{\cdot}1 \times 10^{-3}$ mg. and $+0{\cdot}9 \times 10^{-3}$ mg. S. For the average of four or five analyses this error range will be halved corresponding approximately to $\pm 0{\cdot}5$ mg. S per 100 ml. for the analysis of 0·1 ml. deliveries. This is sufficiently accurate for our present purpose.

Table I.

No. of analysis	Burette reading for titration of benzidine ppt. cm.	Calculated quantity of sulphate mg. S
1	13·1	$10{\cdot}3 \times 10^{-3}$
2	12·7	$10{\cdot}0 \times 10^{-3}$
3	12·7	$10{\cdot}0 \times 10^{-3}$
4	11·5	$9{\cdot}0 \times 10^{-3}$
5	13·1	$10{\cdot}3 \times 10^{-3}$

Blank value = 0·25 cm. on burette; allowed for in the above readings.

The burette contained 0·005 *N* KOH (CO_2-free) and 1 cm. distance on it corresponds to $0{\cdot}77 \times 10^{-3}$ mg. S.

The analyses are the first five of a series of 24 on 0·1 ml. deliveries of a pure sodium sulphate solution containing 10 mg. S per 100 ml. (factor of 0·1 ml. pipette = 0·98).

Standard deviation for the total group of 24 is $0{\cdot}6 \times 10^{-3}$ mg. S.

Experimental procedure. The kidneys of the pithed frog were at once removed to filter-paper, trimmed of extraneous tissue and if not immediately used for analysis were placed in Locke's solution contained in a tube of about 5 ml. capacity. Oxygen saturated with water vapour was bubbled through rapidly so that the kidneys were constantly stirred, the oxygen being introduced through a fine tube fixed in position with a little sealing-wax. The Locke's fluid was renewed every 10 minutes for half an hour (this renewal was scarcely necessary as the volume of fluid was always sufficiently great—50 to 100 times the volume of the kidneys—to render the diffused material insignificantly small). After half an hour the kidneys were removed and if not immediately dried, weighed and analysed were removed to another similar tube holding some sulphate-Locke solution with or without potassium cyanide (0·02–0·04 %). The oxygen bubbling was then carried out as before, the kidneys being removed for analysis after the required interval.

In obtaining samples of blood for analysis the frog was pithed and a cannula quickly inserted into one carotid. Sufficient blood could be obtained as a rule in small tubes of 0·5 ml. capacity with pointed ends arranged on plasticine. The blood was allowed to clot, centrifuged and the serum taken for analysis. Urine was taken at the same time from the bladder if this contained any, the bladder being ligatured at both ends, removed and the urine taken.

Results.

The sulphate content of the serum, urine and kidneys of normal frogs (Rana temporaria). Table II gives the results of the analyses of blood and urine from

Table II.

No. of analysis	Serum-sulphate conc. mg. S per 100 ml.	Urine-sulphate conc. mg. S per 100 ml.	Urine conc. as % of plasma conc.
1	7·9	1·9	24
2	8·3	1·5	18
3	5·8	2·0	35
4	5·6	0·3	5
5	7·3	3·1	43
6	8·6	2·8	33
7	3·8	18·3	480

The above analyses were made in April, 0·1–0·4 ml. of serum or urine being used.

the same frog. Fig. 1 gives the distribution of the total analyses of sulphate in serum, kidney and urine. The mean concentration of sodium sulphate in the

frog's serum from 19 estimates is 6·2 mg. S per 100 ml. with a standard deviation of the mean of 0·4 mg. S per 100 ml. The distribution may be expressed by the standard deviation for the single analysis, namely 1·8 mg. S per 100 ml. (If we allowed for the distribution produced by the error of estimate this would show only a small reduction to 1·7 mg. S per 100 ml.) The results for the urine analyses fall into two groups, one containing the bulk of the observations and showing a marked absorption and a small group showing an equally marked secretion of sulphate. For the group of urine analyses (18 in number) fully carried out, four are much in excess of the usual blood figures. These urines are at once distinguished by the thick benzidine precipitates they cause and in

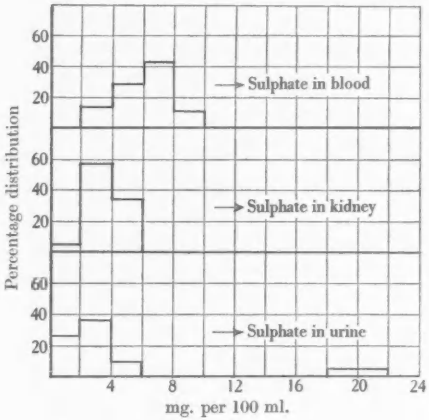

Fig. 1.

this way a number of samples were tested without proceeding fully with the analysis. It was found that rather less than 15 % fall in this group, the proportion varying with different consignments of frogs. For one consignment of 36 summer frogs no high concentration in the urine could be demonstrated. The average urine value for the absorbed group was $2{\cdot}7 \pm 0{\cdot}4$ S per 100 ml., the standard deviation of the individual determination being 1·5 mg. S per 100 ml.

The sulphate content of the normal kidney of the frog was found from 17 analyses to be $3{\cdot}3 \pm 0{\cdot}3$ mg. S per 100 g. or about half that of the plasma. The distribution of the analyses is shown in Fig. 1, the standard deviation of the individual determination being 1·1 mg. S per 100 g. In this figure for the renal sulphate is included 0·4 mg. S per 100 g. which is apparently indiffusible into the surrounding Locke's fluid and though estimated as sulphate, may not in fact be this substance or may have a merely analytical significance. This would leave 2·9 mg./100 g. S as the value of the diffusible inorganic sulphate in the kidney and so represent 47 % of that in the plasma. It will be seen below that about the same figure is reached for diffusion equilibria in sulphate-Locke which contains inorganic sulphate in the concentration of 10–100 mg. S per 100 ml.

The diffusion of sulphate from the isolated normal frog's kidney. The kidneys (usually two) were suspended in Locke's fluid free from sulphate and kept constantly stirred by bubbling oxygen through. The sulphate quickly leaves the kidneys so that in 2 minutes about 36 % of the initial amount has diffused.

In 30 minutes 89 % has diffused and, as previously indicated, further time allowed for the diffusion does not change this average figure.

Equilibrium concentrations of the kidney in cyanide-sulphate-Locke solution ("inactive" kidneys). With the cyanide-sulphate-Locke solution a sulphate equilibrium of 58 % (by weight) of the external fluid is reached in about 15 minutes and this figure is maintained very steadily up to 90 minutes, after which there is a comparatively sharp rise to a new equilibrium representing 74 % of the external sulphate. The experiments illustrated in Fig. 2 were made with a solution

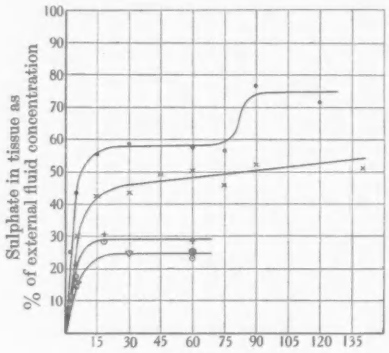

Fig. 2. × Kidney. • Kidney (cyanide). + Liver. ⊙ Liver (cyanide). ▽ Sartorius. o Sartorius (cyanide).

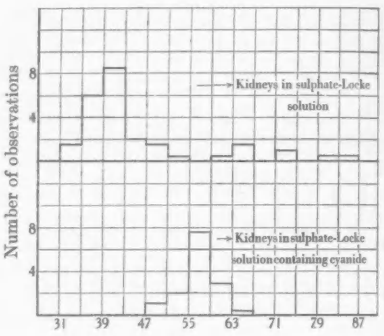

Fig. 3.

Table III.

External sulphate mg. S per 100 ml.	Active kidney: Sulphate conc. mg. S per 100 g.	Active kidney: Sulphate conc. as % of external sulphate	Inactive (cyanide) kidney: Sulphate conc. mg. S per 100 g.	Inactive (cyanide) kidney: Sulphate conc. as % of external sulphate
10	3·6	36 (4)	6·1	61 (4)
20	8·4	42 (4)	—	—
25	9·7	39 (4)	—	—
60	21·0	42 (9)	28·5	57 (9)
100	43·0	43 (4)	59·0	59 (2)

The figures in brackets represent the number of experiments.

The kidneys in all cases were first washed in Locke's fluid for 30 minutes, being stirred by a bubbling stream of oxygen. Subsequently the kidneys termed "active" were immersed in a sulphate-Locke fluid for 30 minutes and the "inactive" kidneys in a cyanide-sulphate-Locke fluid. The kidneys were in all cases stirred by a stream of oxygen.

containing sulphate in the strength of 50 mg. S per 100 ml. Varying the sulphate content of the external fluid (from 10 to 100 mg. S per 100 ml.) produces no change in the equilibria in the kidney when expressed as percentages of the external sulphate (see Table III). Fig. 3 shows the distribution of 12 individual analyses for the cyanide equilibria with external sulphate 20–50 mg. S per 100 ml. The mean of these cyanide values is 58, the standard deviation of the individual analyses being 3·7. (The figure shows also the distribution of values for the active kidney under similar conditions.)

Equilibrium concentrations of the kidney in sulphate-Locke solutions ("active" kidneys). Here as with the inactive kidneys the diffusion process tends to completion in about 15 minutes, though there is a subsequent slow rise which is obviously due to a shifting of the equilibrium value itself. From zero time onwards the sulphate content of the inactive kidney immersed in sulphate-Locke considerably exceeds that of the active kidney as may be seen from Fig. 2. The equilibrium values in Fig. 2 for active kidneys in sulphate-Locke were taken from the data obtained from a single large consignment of local frogs in June, the sulphate-Locke solution containing in all cases 50 mg. S per 100 ml. as sodium sulphate. The conditions were therefore uniform while the time of immersion was varied. (The results of previous experiments had shown an even lower figure for the equilibrium of the active kidneys of consignments earlier in the year.) From 5 to 30 minutes after immersion the increase of the sulphate concentration in the inactive (cyanide) kidney over that of the active amounts to 13–16 % of that of the external sulphate or 43–30 % of the actual sulphate concentration in the active kidney (see Table IV). As time proceeds the difference diminishes as may be seen from Fig. 2. The difference also appears at all concentrations (10–100 mg. S per 100 ml.) in the external sulphate-Locke, as shown in Table III.

Table IV.

Tissue	Period of immersion in sulphate-Locke mins.	Normal tissue: Conc. as % of external sulphate. Mean value	Normal tissue: No. of exps.	Tissue in cyanide: Conc. as % of external sulphate. Mean value	Tissue in cyanide: No. of exps.	Increase in conc. of tissue over normal as % of external fluid
Frog's kidney	5	30	4	43	4	+13
	15	43	4	56	2	+13
	30	43	2	59	4	+16
	60	50	4	58	1	+ 8
	75	46	4	56	4	+10
Frog's muscle (sartorius)	2·5	10·7	4	9·0	4	− 1·7
	5	14·4	5	13·3	6	− 1·1
	10	17·1	4	15·5	2	− 1·6
	30	25·6	3	—	—	—
	60	24·6	3	24·8	6	+ 0·2
Frog's liver	5	20·4	3	16·1	2	− 4·3
	18·5	30·3	2	27·5	2	− 2·8
	60	27·6	3	23·0	2	− 4·6

Before immersion in sulphate-Locke or in sulphate-cyanide-Locke all tissues were washed in Locke for 30 minutes, being stirred by a stream of oxygen. The experiments were carried out at room temperature which varied from 20 to 25°. The experiments were performed in June and July.

When the external sulphate-Locke solution contains 10 mg. S per 100 ml., the equilibrium figure (after 30 minutes' immersion) appears lower than that for higher concentrations. With the colorimetric method this difference for the lower concentrations in the external fluid was even more marked.

It was previously noted that occasionally the urine-sulphate showed a much higher concentration than the plasma-sulphate, and that this occurred in about 15 % of the total number of samples examined. It might be expected that the "active" kidneys would also occasionally show evidence of a "positive" secretion of sulphate rather than a "negative" one.

This would appear in a higher equilibrium concentration in the active kidney than in the inactive, and this was found in a small proportion of the analyses. In 82 analyses (21 colorimetric) made on the active kidney 14 were markedly higher than for the inactive kidneys.

The distribution of the analyses for the active kidney is shown in Fig. 3, where the conditions are similar—30 minutes' immersion in the sulphate-Locke solution of kidneys from frogs of the same or similar consignments and 20–50 mg. S per 100 ml. in the external Locke fluid. Over this range of concentration it has been shown that no appreciable difference exists in the relative value of the equilibrium concentration. This value tends to a figure representing 41 % of the external sulphate, the mean value—considering only the kidneys which are "diluting" sulphate—is also 41 and the standard deviation of the single determination 4·4.

The proportion of times in which the high equilibria for the active kidney occurs is the same as that for the occurrence of high urine values.

Sulphate diffusion equilibria in muscle. The striking differences between the sulphate equilibria for the kidneys in cyanide-sulphate-Locke and in sulphate-Locke led naturally to a consideration of the possibility of similar differences in muscle and other tissues. The sartorius muscle of the frog was selected in the first instance. The dimensions in mm. of the average muscle investigated were $30 \times 3{\cdot}5 \times 1{\cdot}05$, and the average weight 115 mg. We may compare these with the average kidney dimensions for frogs received from abroad in March and April, which were in mm. $16 \times 3{\cdot}0 \times 0{\cdot}7$, and the average weight 35 mg. For local frogs received in May and June the average dimensions were $18{\cdot}6 \times 3{\cdot}5 \times 0{\cdot}8$ mm. and the average weight 55 mg. It will be seen that the figures were somewhat similar for the breadth and thickness of the two tissues. The muscles were treated in a similar manner to the kidneys, receiving a preliminary washing in Locke's fluid for 30 minutes. Analysis of the muscles after this procedure gave an average value of 1·4 mg. S per 100 g. Since with the dimensions of the sartorius and the rate of entrance of sulphate practically all diffusible sulphate should have passed out in 15 minutes this apparent or fixed sulphate was deducted as a blank value. The muscles after this preliminary washing were then immersed in sulphate-Locke or cyanide-sulphate-Locke containing 50 mg. S per 100 ml. Oxygen was bubbled through as before and after varying times the muscles were removed for analysis in the same way as the kidneys. It was found that diffusion equilibria were reached in about the same time as with the kidneys, the diffusion process being nearly complete in 15 minutes. The equilibrium value for the muscle was much lower than for the kidney representing 25 % only of the volume of the muscle. The effect of cyanide was markedly different from that on the kidney. So far from effecting a much higher equilibrium concentration it caused a slight reduction. The results are shown in Fig. 2 and Table IV. The table shows a small reduction in the amount of sulphate entering the muscle due to the action of cyanide which is rather consistent over the whole period investigated (0–60 minutes).

Sulphate equilibria for liver sections. Sections were cut from the frog's liver having the average dimensions $11{\cdot}2 \times 1{\cdot}8 \times 1{\cdot}1$ mm. These were treated in a similar manner to the other tissues. After washing for 30 minutes in Locke's fluid a blank value represented by 3·6 mg. S per 100 g. was found (average of four analyses ranging from 6·6 to 2·7 mg. S per 100 g.). The analysis of two unwashed sections gave 7·2 and 7·3 mg. S per 100 g. As before whatever significance this blank value may have it does not represent freely diffusible sulphate which would have all disappeared in the washing in Locke's fluid. The liver sections were then immersed for varying times in sulphate-Locke (50 mg. S per 100 ml.) in the manner previously described. The results are summarised in Table IV and Fig. 2. The equilibrium values are again practically reached in 15 minutes, the average value for the equilibrium in sulphate-Locke (50 mg. S

per 100 ml.) was 29 % of the external sulphate and 25 % for the sections in cyanide-sulphate-Locke, showing as with the muscle a small reduction due to the cyanide.

Discussion.

It will be seen from the results that for about 85 % of frogs' kidneys (*Rana temporaria*) inorganic sulphate is—in the terminology of renal physiology—a strongly re-absorbed substance and in about 15 % just as markedly secreted. In this we suppose that all the urine volume comes from the glomerulus and corresponds there in composition to a blood-filtrate. In the one instance where a normal blood analysis and a corresponding highly concentrated urine were analysed the high urine concentration was not associated with a high but rather with a low plasma concentration. It is well known that the frog does not concentrate chloride at any plasma figure—so that there is no threshold value for secretion of chloride in the sense in which this is used for mammals, nor is it likely that a secretion threshold exists for sulphate but rather that the kidneys are concentrating or absorbing sulphate in reference to some special condition.

Corresponding to this relationship of urinary sulphate and plasma-sulphate we have the fact that the equilibrium concentration in the active kidney is, in the large majority of cases, considerably less than that of the inactive (or cyanide) kidney, the relationship being about 41 to 58, expressed as percentage of the sulphate in an external Locke-sulphate solution. The relationship is very clearly shown in the frequency distribution given in Fig. 3. The figure shows also that a small number of the equilibrium figures for the active kidney lie well above the region for the inactive (cyanide). These high values may be presumed to correspond to kidneys which "concentrate" rather than "dilute" sulphate.

These results are in accord with a view of renal secretion already propounded [Conway and Kane, 1929]. According to this view the main function of the glomerulus is to take control of the water secretion, the actual secretion and absorption of substances being a diffusion towards potential equilibria created by the active cells. The existence of such potential equilibria must be postulated even with the water absorption theory to explain the much more rapid passage relative to the water of substances such as chloride and glucose across the cells. This passage must inevitably occur towards the end of the absorbing region for urines which are low in chloride or glucose, and it may be emphasised that the passage is then from a low urine concentration to a relatively much higher blood concentration, so that the problem of special secretion is in reality contained in the theory which appears to avoid it, the direction of the process being merely reversed. The potential equilibria considered here are in one sense dynamic in that they necessarily involve the expenditure of energy in bringing a substance such as sulphate from a lower urinary concentration to a higher blood concentration. If a flow of blood were maintained so that there was insufficient time for it to reach equilibrium, then sulphate would pass continuously across the active cells from the flowing urine into the blood-stream. If the blood were stationary, and a sufficient oxygen supply conceivable, the final blood concentration would be higher than that in the cells or in the urine. This condition is simulated by immersing the kidney in an oxygenated sulphate-Locke solution. It will be seen that this implies that for a sulphate-Locke solution of constant composition in which the kidney is immersed the concentration of sulphate in the active organ is less than in the inactive. This is the experimental result obtained and described above.

It will be observed that this explanation need involve no other cardinal assumption than that a glomerular fluid is formed similar in composition to a blood-filtrate. It does not state how the potential equilibria of the active cells are brought about, it merely considers their necessity. It involves the consequence that for secreted substances the opposite result should be obtained, namely that the total concentration of the active kidney at equilibrium should be higher than that of the inactive kidney. As before mentioned this difference has been fully demonstrated in this laboratory for urea, phenol red and bromophenol blue.

SUMMARY.

1. The average concentration of sulphate in the blood-plasma of 19 normal frogs (*Rana temporaria*) was found to be 6·2 expressed as mg. S per 100 ml. The distribution is given by the standard deviation—1·8 mg. S per 100 ml.

2. The urine concentration is in general much lower than that of the blood, approximating to one-third or less of its value. A small group showed the urinary sulphate to be concentrated like urea.

3. The concentration of sulphate in the kidney is likewise much less than in the blood, tending to about 40 % of its value.

4. The sulphate passes freely in and out of the frog's kidney in relation to the concentration of sulphate in a surrounding sulphate-Locke fluid. Equilibrium is practically reached in 15 minutes. For the active kidney the diffusion equilibrium tends to a value of 41 % of that of the external sulphate. For the inactive kidney (cyanide) the equilibrium concentration is 58 % of that of the external sulphate (or 72 % of the total possible concentration with full and equal permeation).

5. The equilibrium concentration in muscle is 25 % by volume of the external sulphate and for liver sections 29 %. Addition of cyanide causes a slight reduction in these figures in marked contrast to its effect on the kidney.

6. The rates at which the equilibrium figure is reached by diffusion are the same for muscle, liver, active kidney or inactive kidney although the equilibrium concentrations differ considerably.

7. The results are in accord with a diffusion-secretion theory of the kidney propounded elsewhere.

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CCXXXIV. DIFFUSION RATES OF ANIONS AND UREA THROUGH TISSUES.

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For diffusion to or from a semi-infinite medium bounded by one plane the following formula may be shown to apply

$$\frac{\text{Amount diffused}}{\text{Surface}} = 2(c_0 - c_1)\sqrt{\frac{kt}{\pi}} \qquad \ldots\ldots(1)$$

where c_0 is a constant concentration in an outside phase and c_1 a uniform concentration in the medium at time t, k being the diffusion coefficient. The formula applies also to a fully bounded medium if t be not too great. It is in fact approximately true up to 70 % of the final value for media of the type of the frog's sartorius which are bounded by parallel planes, and gives values for k which are then theoretically within 5 % of the true figure.

In using equation (1) it is supposed that the equilibrium concentration in the medium is the same as the external concentration c_0. If however the equilibrium concentration is not c_0 but ec_0 where e is a factor, then the above formula must be written

$$\frac{\text{Amount diffused}}{\text{Surface}} = 2(ec_0 - c_1)\sqrt{\frac{kt}{\pi}} \qquad \ldots\ldots(2).$$

The reason for this is apparent if we consider a film of extreme thinness immediately underneath the surface of the medium. The concentration in this film may be regarded as attaining almost instantaneously the concentration ec_0 and the subsequent diffusion therefore takes place as if from this film, the "diffusion head"—as one may express it—being $(ec_0 - c_1)$.

Neglect of this latter consideration leads to an apparently large difference between the diffusion constants of phosphate and lactate anions (at relatively low concentrations) through muscle [Eggleton *et al.*, 1928; Stella, 1928]. This was explained by Eggleton *et al.* as being due to the expansion of the muscle fibres after a long period in Ringer solution. The phosphate diffusion was examined by Stella after 360-minute periods and the lactic acid diffusion by Eggleton *et al.* after 7½–52 minutes. It was supposed that swelling of the fibres (which was presumed to develop late) would diminish the interspace water in which the diffusion was rapid and cause a fall in the diffusion constant. A fall in the diffusion constant for lactate was shown to occur in the fatigued muscle and was attributed to a similar diminution in the lymph interspaces from swelling of the muscle fibres. This swelling should presumably occur from a rise in osmotic pressure inside the fibres. No evidence however was brought for such diminution in the interspace water in Stella's experiments, and furthermore no such diminution even in fatigued muscle could be demonstrated by G. Eggleton [1933] in subsequent experiments. There is in fact no explanation necessary as the diffusion constant for phosphate from the combined data of

Stella [1928], G. Eggleton [1933] and Semeonoff [1931] turns out to be almost identical with that for lactate through muscle when equation (2) is used. Also it will be shown below that sulphate and chloride diffuse in a very similar manner to phosphate and all four anions give the same diffusion coefficient through muscle when equation (2) is used, but widely different values when equation (1) is used.

Where other tissues (kidney, liver) were investigated for sulphate diffusion (in the presence or absence of cyanide) the diffusion coefficient was also the same as in muscle, although it may be noted that the equilibrium values for the diffusion were often very different. This general diffusion coefficient for the anions is, as will be shown, $6{\cdot}0 \times 10^{-5}$ cm.^{2}/min., the standard deviation of the individual estimate being $1{\cdot}3 \times 10^{-5}$.

For comparison with the anion diffusion that of urea was also investigated. As shown below urea has a much slower diffusion rate than that of the anions considered, the value of the coefficient being $1{\cdot}1 \times 10^{-5}$, although urea has a smaller molecular weight than three of these anions.

For convenience of calculation equation (2) may be written in the following form for tissue diffusion:

$$\text{Ratio of equilibrium value reached} \times \frac{\text{Weight in g.}}{\text{Surface in cm.}^2} \times 0{\cdot}843 = \sqrt{kt}.$$

This is derived from equation (2). (In the derivation it is assumed that concentration per unit weight of tissue may be converted into concentration per unit volume by multiplying by 1·05.)

The weight to surface relationship may be read off from curves for the individual tissue, or derived from the following formulae for the sartorius or kidney of the frog:

$$\frac{\text{Weight}}{\text{Surface}} = \text{Wt.}^{\frac{1}{3}} \times 0{\cdot}0844 \text{ (sartorius)}.$$

$$\frac{\text{Weight}}{\text{Surface}} = \text{Wt.}^{\frac{1}{3}} \times 0{\cdot}0858 \text{ (kidney)}.$$

Methods of investigation.

The general procedure was the same as that described in the previous communication. The sartorii, kidneys or liver sections from the frogs were washed for 30 minutes as a rule in Locke's fluid and then immersed in sulphate-Locke, being stirred by a stream of oxygen. The rate of emergence of sulphate from the freshly excised kidney was also investigated. The analytical procedure for sulphate was the same as that previously described.

The chloride diffusion into the sartorius muscle was examined in the following manner. For each experiment four frogs were taken and a sartorius from each removed for analysis—the four sartorii so taken being analysed in bulk. The four twin sartorii were then immersed in Locke's fluid, containing however 0·7 % of sodium chloride, and stirred with oxygen for 2–5 minutes when they were removed, dried at once on filter-paper, the excess fluid being removed by gentle pressure between filter-papers, and then taken for analysis. The equilibrium values were obtained by immersion of other groups of muscles for 30- or 60-minute periods in the same Locke fluid, the frogs being taken from the same consignment. The chloride analyses were carried out (after weighing) by firstly grinding in an agate mortar with 1 ml. of water with the addition of a little of the purest quartz sand. The grinding was sufficient to reduce the muscle to a thin paste. 1 ml. of 3·0 % sodium tungstate was now added and stirred and subsequently 1 ml. of 0·2 N sulphuric acid. The same proportions of tungstate

and sulphuric acid were therefore used as in the well-known Folin method [Folin and Wu, 1919] of blood analysis. The mixture was transferred to a 15 ml. centrifuge-tube and centrifuged for a short time. 2 ml. of the clear fluid were taken for the chloride analysis which was carried out as follows. The chloride was converted entirely into chlorine at room temperature by a suitable acid-permanganate mixture in the outer chamber of the special absorbing unit previously described [Conway, 1933], the chlorine being absorbed in 20 % potassium iodide in the central chamber of this apparatus. The iodine liberated after full absorption of the chlorine ($1\frac{1}{2}$ hours) was titrated with standard thiosulphate using most conveniently the micro-burette described in a previous communication [Conway, 1934]. Suitable controls were carried out in all cases.

Sulphate diffusion into tissues.

After a preliminary washing of excised tissues in ordinary Locke for 30 minutes the values for sulphate found on analysis ranged from 0·4 mg. S per 100 g. in the kidney to 4·0 mg. per 100 g. S in the liver. Prolonged washing did not change this value for the kidney and it was considered—as noted in the last communication—that these values very probably do not represent free inorganic sulphate and have only an analytical significance. They were treated as blank values and the true concentration of free inorganic sulphate in the tissues after 30 minutes' washing was taken as zero. Subsequently to this preliminary washing the tissues were immersed in sulphate-Locke solution containing 50 mg. S—or 1·56 millimols—per 100 ml. Equilibrium with respect to sulphate is practically reached after 15 minutes as shown in the previous communication. Up to 5 minutes equation (2) may be used for calculating the diffusion coefficient, there being no appreciable fall from the value obtained at 2 minutes as may be seen from Table I (for chloride and sulphate). For muscle

Table I.

Tissue	Substance	No. of obs.	Average weight in mg.	e	Diffusion head $(ec_0 - c_1)$ $(\times 10^2)$	t mins.	Mean % of equil. value reached	Mean value of k $(\times 10^5)$
Kidney (in cyanide)	Sulphate	4	61	0·58	0·90	2·0	40	6·7
Sartorius	Chloride	4	67	0·30	2·73	2·0	35	5·6
Sartorius	Sulphate	4	93	0·25	0·39	2·5	38	5·4
Kidney	Sulphate	4	48	0·58	0·90	5·0	73	6·5
Sartorius	Sulphate	5	92	0·25	0·39	5·0	56	6·9
Sartorius	Chloride	2	70	0·30	2·73	5·0	56	6·0

In the table e is the equilibrium concentration (per unit volume) reached expressed as a ratio of the external concentration. c_0 and c_1 are the external concentration and the tissue concentration at zero time expressed as millimols per 100 ml. (For the calculation of the diffusion coefficient it is immaterial whether we express quantities as g. or millimols.)

the diffusion coefficient calculated after 5 minutes would seem greater than after 2·5 minutes but the difference is within the sampling error. Table II gives a summary of the data obtained for sulphate diffusion with the data for other anions. The mean value of 39 estimations of the diffusion coefficient of sulphate through tissues at room temperature (20°) is $6{\cdot}3 \times 10^{-5}$, or $6{\cdot}1 \times 10^{-5}$ omitting two aberrant results, the standard deviation of the individual estimate being then $1{\cdot}3 \times 10^{-5}$. The diffusion coefficient was found to be independent of the tissue investigated whether muscle, liver or kidney.

Table II.

Tissue	Substance	No. of obs.	Millimols per cm.^{2} $\frac{}{\sqrt{t}}$ ($\times 10^5$)	$(c_0 - c_1)$ millimols per 100 ml.	e	$(ec_0 - c_1)$ millimols per 100 ml.	k from equation (1) ($\times 10^5$)	k from equation (2) ($\times 10^5$)
Sartorius	Chloride	6	23·5	12·1	0·30	2·73	0·27	5·7 (3·0–8·2)
Kidney (in cyanide)	Sulphate	8	8·2	1·56	0·58	0·90	2·22	6·6 (5·0–10·7)
Kidney	Sulphate	4	6·1	1·56	0·45	0·70	1·21	6·0 (5·8–6·7)
Liver	Sulphate	3	3·8	1·56	0·29	0·45	0·47	5·6 (5·0–6·6)
Liver (in cyanide)	Sulphate	2	3·3	1·56	0·25	0·39	0·36	5·7 (6·2–5·2)
Sartorius	Sulphate	9	3·3	1·56	0·25	0·39	0·39	6·3 (2·9–13·5)
Sartorius (in cyanide)	Sulphate	6	3·2	1·56	0·23	0·36	0·33	6·3 (5·5–7·5)
Leg muscle	Phosphate	8	0·66	—	0·29	0·075	0·51	6·0
Kidney	Sulphate	7	−0·082	−0·091	—	−0·091	6·4	6·4
Leg muscle	Phosphate	8	−3·43	—	0·29	−0·393	0·51	6·0
Leg muscle	Lactic acid	8	−8·9	−1·05	—	−1·05	5·6	5·6 (3·2–6·9)

The data for phosphate diffusion were taken from Stella [1928], the values at the ends of his curve being taken as illustrating the widest positive and negative diffusion for resting muscle investigated by him in that communication. The e value for phosphate, 0·29, has been discussed in the text. The data for the lactic acid diffusion were taken from the figures of Eggleton *et al.* [1928]. The k value in the penultimate column for phosphate is Stella's calculation.

Diffusion of sulphate from tissues.

This was investigated for the freshly excised kidney. A number of such kidneys were analysed for sulphate and others from the same batch of frogs were washed in Locke's fluid for varying periods and subsequently analysed. A summary of the data is given in Table III. The average value for sulphate

Table III.

Time mins.	Sulphate in kidney. Mean values as mg. S per 100 g.	No. of analyses	Average weight of tissue analysed mg.	Average weight of kidney mg.
0	3·3 ± 0·3	17	155	42
2	1·9 ± 0·3	7	111	57
30	0·4	2	166	42
60	0·3	2	145	36

in the normal kidney is 3·3 ± 0·3 mg. S per 100 g. (mean of 17 observations). After 2 minutes' washing this is reduced to 1·9 mg. S per 100 g. (mean of 7 observations, average kidney weight being 57 mg.). After prolonged washing the value is 0·4 mg. S per 100 g. This as before mentioned is considered as a blank value. From the data in Table III the diffusion coefficient is calculated as $6{\cdot}4 \times 10^{-5}$, or practically the same as that for the entrance of sulphate. This in itself provides an interesting example of the validity of equation (2) in calculating diffusion coefficients through tissues. For diffusion of sulphate from the tissues into Locke's fluid it is immaterial whether we use equation (1) or (2) since the external solution has practically zero sulphate concentration. We get

then with either formula $6{\cdot}4 \times 10^{-5}$. For diffusion inwards on the other hand if we used equation (1) we should obtain $0{\cdot}95 \times 10^{-5}$ whereas with equation (2) we get $6{\cdot}1 \times 10^{-5}$. From this illustration alone the correctness of equation (2) is apparent since the diffusion rates in either direction must be identical (except in the special case which does not arise here of a large internal storage).

Diffusion of chloride into muscle.

The manner in which this was carried out has been described above. The large number of data for sulphate diffusion were collected in the course of the previous investigation. In regard to chloride it was considered sufficient here to demonstrate its diffusion coefficient through muscle. Table IV gives a summary

Table IV.

NaCl at zero time g. per 100 g.	NaCl after immersion in Locke containing 0·74 % NaCl g. per 100 g.	Time of immersion in minutes	Average weight of single sartorii after immersion mg.
0·051	0·109	2	72
0·044	0·109	2	79
0·065	0·124	2	62
0·070	0·110	2	57
0·082	0·150	5	72
0·053	0·157	5	69
0·057	0·232	30	47
0·061	0·221	30	42
0·051	0·227	30	45
0·061	0·220	30	61
—	0·220	60	53
—	0·207	60	43

The analyses of the first two columns are for twin sartorii, two sartorii from two frogs being analysed after excision and the remaining two after immersion for varying periods in Locke solution containing 0·74 % NaCl at room temperature.

of the data obtained. The mean value of the six sets of determinations of the diffusion coefficient (for the 2- and 5-minute periods) is $5{\cdot}7 \times 10^{-5}$. This value is thus the same as that for sulphate within the limits of sampling error. The distribution of the individual values is also similar. The value obtained from equation (1) is $0{\cdot}27 \times 10^{-5}$.

It may be noted also that the chloride diffuses from the external Locke fluid into the muscle until it reaches an equilibrium figure 30 % of that of the external chloride. The sulphate diffused up to 25 %. The average value for the chloride in the frog's muscle as taken directly from the frog was found to be 0·060 %, expressed as sodium chloride. After 30 or 60 minutes' immersion in the Locke fluid containing 0·74 % total chloride the average value was 0·221 %.

Comparison of the rates of chloride and sulphate diffusion with those of lactic acid and phosphate.

The data for lactic acid diffusion were taken from the paper by Eggleton *et al.* [1928]. In their technique frog's legs were skinned, stimulated in nitrogen and then immersed for the diffusion period or periods in bicarbonate-buffered oxygen-free Ringer solution, the amount diffused being determined by analysing the fluid, the muscles themselves being finally analysed for lactic acid. It is immaterial whether we use equation (1) or (2) in determining the k value for lactic acid here as the external concentration is zero. The diffusion coefficient

falls after a lactic acid concentration of 1·08 mg. per ml. (In Table I of their paper the authors have given the percentage values of lactic acid in muscle instead of the mg. per ml. mentioned at the head of the column.) The authors have summarised the k value for low initial concentrations as "about $6{\cdot}0 \times 10^{-5}$." The diffusion coefficient of lactate is therefore practically identical with those for sulphate and chloride.

The values for phosphate diffusion have been taken from the data of Stella [1928]. He immersed frogs' legs in Ringer containing varying quantities of phosphate and measured the phosphate diffused in or out of the muscle after 6-hour periods. The diffused amounts were calculated from the analyses of the Ringer's fluid. When the concentration of phosphate in the Ringer fluid is such that no diffusion of phosphate occurs into or out of the muscle then the diffusion head $(ec_0 - c_1)$ must be zero, c_0 being the concentration in the Ringer fluid and c_1 in the muscle, e being a factor already considered. If we consider any change from this particular phosphate concentration in the Ringer fluid, then we may write the diffusion head or effective diffusing concentration as ec' where c' is the change of this Ringer-phosphate concentration. The values of e from the figures given by Stella for prolonged immersion are as high as 0·55. G. Eggleton [1933] has pointed out that such are certainly too high owing to the diffusion of phosphate from the bone. From her data the e value would be 0·21. From Semeonoff's data [1931] on the isolated sartorius the e value is 0·38. Taking the mean of Eggleton's and Semeonoff's figures e is 0·29. This is close to the values obtained by us for sulphate and chloride, namely 0·25 and 0·30 respectively, and thus has this added probability of being the most representative figure. Using this value of e and Stella's data for phosphate diffusion in muscle, from equation (2) we get $6{\cdot}0 \times 10^{-5}$ as the corrected value for the diffusion coefficient. This value is again the same as those of the anions previously considered.

For the four anions, chloride, sulphate, lactate and phosphate, we have then the mean values of the diffusion coefficient through the tissues of the frog at room temperature, $5{\cdot}7 \times 10^{-5}$, $6{\cdot}1 \times 10^{-5}$, $5{\cdot}6 \times 10^{-5}$ and $6{\cdot}0 \times 10^{-5}$, which values differ from each other no more than one might expect from the sampling error. It may also be noted that diffusion coefficients are proportional to the square of the amount of substance diffused, and any error or divergence in this is therefore of considerable influence in the calculated coefficient.

The diffusion of sulphate was examined for three different tissues, sartorius, liver and kidney, the mean values being $6{\cdot}1 \times 10^{-5}$, $5{\cdot}6 \times 10^{-5}$ and $6{\cdot}0 \times 10^{-5}$, which are again the same within the expected error of sampling. It is also immaterial whether the sulphate diffuses inwards to 58 % of the external concentration as with the kidney in cyanide or to 23 % as with the sartorius in cyanide; the diffusion coefficient remains the same, *viz.* $6{\cdot}6 \times 10^{-5}$ and $6{\cdot}3 \times 10^{-5}$ respectively. We are therefore concerned here with a general diffusion coefficient for anions which remains the same under a variety of conditions.

The equivalence of these k values for the different anions turns on the use of the factor e and equation (2)—the use in other words of the conception of the diffusion head as $(ec_0 - c_1)$ and not as $(c_0 - c_1)$. The validity of this conception is again apparent from Fig. 1 where the amount diffusing per cm.^{2} divided by the $\sqrt{t}$ is plotted against the values of $(ec_0 - c_1)$. In introducing $\sqrt{t}$ we are in effect considering the amount diffused in the first unit of time reckoning from the beginning of the diffusion period. The amounts diffused then in this first unit of time and through unit surface lie closely along a straight line passing through the zero point—as shown in Fig. 1. The points in the figure are for three different tissues and four different anions. The quantities diffused plotted

against the values of $(c_0 - c_1)$, on the other hand, show no relationship whatever as may be gathered from an inspection of the penultimate column in Table II, which also gives the data from which Fig. 1 has been drawn.

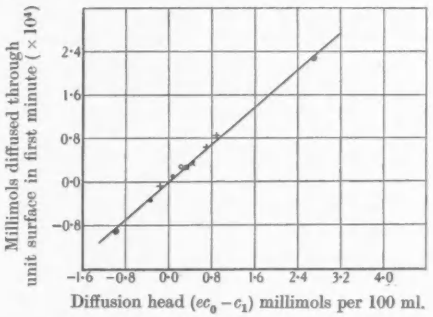

Fig. 1. The diffused quantities of the four anions per unit surface and time when plotted against the diffusion head $(ec_0 - c_1)$ lie closely on a line which passes through the zero point. The same values plotted simply against the difference between the internal and external concentrations $(c_0 - c_1)$ show no relationship whatever.

⊙ Chloride through muscle.
\+ Sulphate through kidney.
× Sulphate through liver.
o Sulphate through muscle.
• Phosphate through muscle.
⊕ Lactate through muscle.

The diffusion of urea through the frog's muscle (sartorius) and kidney.

The method for analysing the urea has been described in detail in a previous communication [Conway, 1933]. For the present purposes it has been extended to the analysis of urea in the tissues. Certain discrepancies have been found between the results of the xanthydrol method and the urease method in the analysis of suspensions of kidney tissue [Rehberg and Clem, 1932; Kirk, 1933]. The experiments to be described show in the clearest manner that all the urea in the kidney as determined by the method used here exists in the same freely diffusible condition as in muscle and that all the urea in muscle is present in free solution, diffusing into and out of muscle at the same rate and having the expected equilibrium value in the tissue-water. We do not consider it essential therefore to discuss in detail in the present communication the alternative methods or their results, but to put forward what we consider to be the conclusive nature of the present findings.

Method. In the analytical method used the kidneys or sartorii, usually about 50–300 mg. in weight, were removed to a small agate mortar and ground with 3 ml. of water accurately measured, a little of the purest quartz sand (Merck's) being used to facilitate the grinding of the tissue quickly to a fine paste. The mixture was then centrifuged, 1 ml. taken for the urea analysis and 1 ml. for the ammonia analysis. It may be noted that in this centrifuging and removal of an aliquot part of the clear fluid we have at least one important difference in technique from the urease method as used by Rehberg, who analysed the tissue suspension without centrifuging. It may be readily shown that the diffusion of urea from the particles of the suspension is a matter only of some seconds and the kidney suspension was thoroughly ground for a few minutes. The 1 ml. of the clear fluid taken for analysis was transferred to the outer chamber of the absorbing unit previously described [Conway, 1933], the

inner chamber containing 1 ml. of 0·001 *N* sulphuric acid. 0·5 ml. of a glycerol-water extract of jack bean diluted with a phosphate buffer solution was added to the outer chamber and incubated for 15 minutes. 1 ml. of saturated potassium carbonate was added to the fluid in the outer chamber in the manner already described and the unit incubated at 37°, the acid in the inner chamber being titrated after 75 minutes, most conveniently with the special burette described in a previous communication [Conway, 1934]. The 1 ml. portion used for the ammonia analysis was treated in an exactly similar manner without the addition of the urease solution. Suitable controls and blanks were carried out in all experiments.

As the amount and concentration of the urease used appear to be of importance in relation to the question of the extra ammonia coming from the enzyme preparation itself, as indicated by preliminary experiments carried out in this laboratory, the constitution of the extract used in the experiments described above was 10 g. of jack bean meal to 50 ml. of glycerol and 17 ml. of water, the whole diluted 20 times with a dilute phosphate buffer solution, 0·5 ml. of this diluted solution being used to about 20–100 mg. of tissue contained in 1 ml. of a centrifuged suspension. With this method and urease solution all the urea analysed in kidney or muscle was of a freely diffusible kind and passed out of the tissue at exactly the same rate as free urea in solution diffused inwards.

Urea in the blood and tissues of normal frogs.

As shown by Marshall and Crane [1924] the concentration of urea in the normal frog's kidney and liver far exceeds that in the sartorius or that in the blood. (This was also demonstrated by Höber [1932] for the perfused kidney.) The absolute quantities of urea vary in different consignments of frogs. We are not concerned however with giving a very general average so much as with the relative quantities. For one consignment of frogs 12 corresponding analyses of the blood, sartorius, kidney and urine were carried out. Neglecting one of these sets in which the blood result is aberrant, we have for the series the following mean values in mg./100 ml.

Blood (whole)	...	...	...	45 ± 2·5
Sartorius	...	...	...	41 ± 2·8
Kidney	...	...	...	85 ± 3·6
Urine	...	...	...	140 ± 12·1

The *plus* and *minus* values give the probable errors of the means. The ratios of the means in the above order are 1·0 : 0·91 : 1·89 : 3·21. (Practically identical ratios are reached if for each set we divide the blood value into the others and take the means of these results.) The value for the sartorius is such as we may expect to be reached by passive diffusion equilibrium between the muscle-water and that of the blood.

Another group of five sets was carried out on corresponding samples of blood, sartorius, kidney, urine, liver and bile, giving the following series expressed as before in mg./100 ml.

Blood (whole)	...	...	53	(39– 65)
Sartorius	...	...	48	(38– 52)
Kidney	...	...	97	(76–100)
Urine	...	...	160	(106–228)
Liver	...	...	147	(118–170)
Bile (gall-bladder)	...	...	225	(57–440)

(The figures in brackets give the range.) The ratio of the blood-urea to that in the other tissues is here again approximately the same. The liver and bile are even richer than the kidney and urine in urea. As before the sartorius has a mean value 0·91 times that of the blood.

The diffusion of urea through the frog's muscle.

In this diffusion we must clearly distinguish between the rate at which urea diffuses and the final equilibrium value reached. The latter is such as we may expect from the urea diffusing freely into all the water of the tissues. This has been shown previously by P. Eggleton [1930] for Ringer-urea solutions in which sartorii muscles were immersed. He determined the urea as nitrogen using the companion sartorius for a correction in regard to other non-protein-nitrogen. Taking the water of the muscle as 80 % by weight the mean value of the urea concentration at equilibrium in this water from Eggleton's data is $1{\cdot}02 \pm 0{\cdot}05$, expressed as a multiple of the external concentration. This corresponds with the results obtained by us in respect to the equilibria between normal blood-urea in the frog and that in the sartorius. We may take it then that the urea in muscle reaches a passive equilibrium with the urea in an external solution, the concentration in the tissue-water being the same as in the external fluid. (This same relation also holds we find for the normal frog's kidney when immersed in cyanide-Locke solution containing urea. It does not hold for the active isolated kidney in which the equilibrium concentration may greatly exceed that of the external fluid.)

Since the water in the muscle occupies about 84 % of the total volume, the analytical results at equilibrium will give an e factor of 0·84. In such a case we may use equation (2) with this e value and regard the diffusion head as $(ec_0 - c_1)$ where c_0 is the external concentration and c_1 the muscle concentration. Strictly however the diffusion head is rather $\left(c_0 - \frac{c_1}{e}\right)$ when we consider the diffusion into the water of the tissue. At the same time we must regard the diffusing surface not as the whole measurable surface but rather as this value multiplied by e. We have then for the diffusion equation

$$\frac{\text{Amount diffused}}{\text{Surface} \times e} = 2\left(c_0 - \frac{c_1}{e}\right)\sqrt{\frac{kt}{\pi}},$$

or

$$\frac{\text{Amount diffused}}{\text{Surface}} = 2\,(ec_0 - c_1)\sqrt{\frac{kt}{\pi}},$$

which latter has again the form of equation (2). For all such cases therefore this equation is valid.

In the experiments for investigating the diffusion coefficient of urea one of the sartorii or one of the kidneys from one or more frogs was transferred to Locke's fluid (or to a cyanide-Locke fluid) and stirred with a stream of oxygen in the manner already described. Such tissues were analysed after varying periods of time. The companion sartorii or kidneys were analysed immediately after excision. In some experiments both sartorii or both kidneys were washed for a preliminary period in Locke's fluid and one was then transferred to a urea-Locke fluid, the other being analysed for urea. In such experiments the diffusion occurs from without inwards. The results are given in Table V. The diffusion of urea is a much slower process than that of lactate, phosphate or sulphate. As before mentioned equation (2) is applicable up to about 70 % of the equilibrium value. This however is not reached for urea until after 30 minutes or more with the tissues examined. Fig. 2 gives the values of the amount of urea diffusing

Table V.

No. of frogs used	Mean urea % at zero time (mg. per 100 g.)	Time of diffusion mins.	Mean urea % after diffusion (mg. per 100 g.)	Mean % of equilibrium reached	Mean weight in mg.	Amount diffused per cm.² / Diffusion head ($\times 10^2$)	k from equation (2) ($\times 10^5$)
Tissue: Kidney:							
7	138	60	21	75	39	2·18	—
2	145	30	45	66	56	2·11	1·16
3	100	20	39	61	40	1·77	1·23
3	86	10	61	29	55	0·96	0·72
6	87	3·6	68	22	45	0·68	1·00
Kidney (in cyanide):							
8	110	60	21	81	41	2·43	—
5	100	20	38	62	42	1·86	1·36
4	155	10	98	37	29	0·96	0·72
7	129	3·3	99	23	47	0·71	1·19
Kidney (in cyanide and 0·08 % urea after 30 mins. washing (in ordinary Locke solution)):							
4	27	30	53	61	65	2·00	1·02
Sartorius:							
5	66	15	42	36	83	1·33	0·83
4	52	30	22	57	113	2·34	1·38
Sartorius (in 0·2 % urea after preliminary washing):							
4	38	5	66	21	130	0·90	1·14
2	28	60	128	70	104	2·80	1·09

With the kidneys the individual tissue was analysed separately, a single kidney from one frog being analysed after excision and its companion after the diffusion period. With the sartorii, single sartorii from the number of frogs mentioned in the second column were analysed together, duplicate analyses being made and the companion tissues being similarly treated after the diffusion period.

In the penultimate column quantities are reckoned in millimols and the diffusion head in millimols per ml.

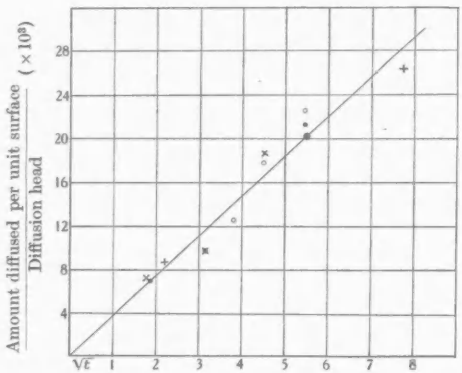

Fig. 2. See text. The diffusion of urea from muscle and kidney follows the square root relationship and the values for both tissues lie along the same line.

• Kidney freshly excised. ⊗ Kidney washed and transferred to a cyanide solution.
▫ Muscle freshly excised in Locke. × Kidney freshly excised in cyanide.
\+ Muscle washed and immersed in urea-Locke.

per cm.^{2} surface of muscle and unit diffusing concentration (unit diffusion head) against $\sqrt{t}$. The square root relation clearly holds. Fig. 3 is of considerable interest. It shows the amount of urea diffusing per unit surface in the first unit of time (obtained by dividing by the square root of the observed time) against the diffusion head of urea. The results lie along a straight line passing through the zero point. This means that the diffusion into or out of the muscle proceeds at the same rate—an expected result in this case.

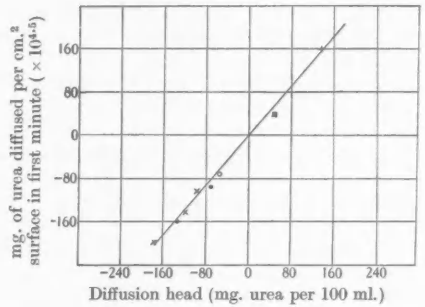

Fig. 3. The diffused quantities of urea per unit surface and time—in or out of the tissues—lie on a straight line when plotted against the diffusion head. This line passes exactly through the zero point, and both muscle and kidney values lie along it. The significance of these results is discussed in the text.

- • Kidney freshly excised in Locke.
- × Kidney freshly excised in cyanide-Locke.
- ⊗ Kidney washed and transferred to a cyanide-urea-Locke solution.
- o Muscle freshly excised in Locke.
- \+ Muscle washed and immersed in urea-Locke.

The diffusion of urea through the frog's kidney.

When however we consider similar values (amounts per unit surface *etc.*) for the freshly excised kidney of the frog in which the urea concentration is about double that of the muscle or blood, the figures lie along the same line as for muscle. So also do the values for the urea diffusion into or out of the kidney immersed in cyanide. The diffusion experiments on the kidney were carried out in a similar manner to those on muscle. Collecting the data for the diffusion coefficients we have:

Tissue	Diffusion coefficient ($\times 10^5$) (from equation (2))
Kidney (freshly excised in Locke)	1·03
Kidney (freshly excised in cyanide-Locke)	1·09
Kidney (washed for 30 mins. in Locke and transferred to a cyanide-urea-Locke solution)	1·02
Sartorius (freshly excised)	1·10
Sartorius (washed for 30 mins. in Locke and transferred to a urea-Locke solution)	1·11

These figures are summarised from Table V. Using the correct form of the diffusion equation (equation (2)) no significant difference exists between the diffusion coefficients of urea diffusing either from the freshly excised active or inactive kidney or into the inactive kidney or to and from the sartorius muscle. The urea in the freshly excised kidney is however about twice that in the blood or in the sartorius muscle. All this "excess" urea diffuses out from the kidney as

fast as it diffuses into the inactive kidney when the equilibrium value is that to be expected in a purely passive equilibration.

We may combine these findings with those in Fig. 3 where the amount of urea diffusing in the first unit of time and per cm.^{2} of surface is plotted against the diffusion head. The figures lie around a straight line passing from positive to negative values through the zero point, there being no apparent difference between the muscle and renal points. We may take it then that the following points are proved by these experiments.

(1) The "excess" urea over the blood value in the frog's kidney as determined by the above method exists in a form as freely diffusible as the urea from an external fluid and as the urea in muscle.

(2) No appreciable amount of this excess urea as above determined is bound in the active kidney.

(3) The freely diffusing urea concentration in the frog's kidney freshly excised has here been shown to be about twice that of the blood value.

The significance of these results for tissue diffusion and renal theory need scarcely be emphasised. The experiments are simple and readily reproducible. It may be noted that the diffusion coefficients are proportional to the square of the amount diffused so that any variation in this quantity is very much increased in the calculation of the coefficient. The similarity of the figures obtained is hence all the more striking.

Discussion.

At the outset we may define for convenience of description and in an empirical manner a term "permeation." By this is meant here that fraction of the total concentration in a tissue which would be reached if there were no barrier to free solution in the tissue water. For lactic acid formed within the muscle fibre, for example, this permeation is 100 %, but for sulphate entering muscle from an external sulphate-Locke solution it is 25/0·84 or 30 % (25 being the percentage equilibrium concentration of the tissue with respect to the external concentration and 0·84 the proportion of water in one volume of tissue). We may make a list of the following permeations with the corresponding diffusion coefficients.

	Permeation	Diffusion coefficient
Lactate (muscle)	100	$5{\cdot}6 \times 10^{-5}$
Sulphate (kidney in cyanide)	69	$6{\cdot}6 \times 10^{-5}$
Sulphate (kidney)	54	$6{\cdot}0 \times 10^{-5}$
Chloride (sartorius)	36	$5{\cdot}7 \times 10^{-5}$
Sulphate (liver)	35	$5{\cdot}6 \times 10^{-5}$
Phosphate (leg muscles)	35	$6{\cdot}0 \times 10^{-5}$
Sulphate (liver in cyanide)	30	$5{\cdot}7 \times 10^{-5}$
Sulphate (sartorius)	30	$6{\cdot}3 \times 10^{-5}$
Sulphate (sartorius in cyanide)	28	$6{\cdot}3 \times 10^{-5}$

The diffusion coefficient is thus independent of the degree of "permeation" (and it may be noted that the square of this value will enter into its calculation). From this it must follow that the interspace water has practically no influence on the observed diffusion rate. The diffusion rate of the anions through this interspace water is no doubt the same as through water itself, that is to say about ten times the observed rate through the tissues. With a permeation of 28 the interspace water must account for a considerable proportion of this and with a permeation of 100 for a comparatively small proportion. Yet for both these permeations the diffusion rate is nearly the same. We are forced therefore

to the conclusion that at least for these excised tissues the tissue elements govern the rate of diffusion and that all diffusion must be primarily conducted through them. The interspace water in such tissues would then exercise very little influence on the observed rate even if the velocity through such interspace water were practically infinite.

If the anion diffusion must pass through the tissue elements which are in contact how is the fall in the diffusion rate of lactic acid with rising concentration in stimulated muscle to be accounted for? This result was obtained by Eggleton *et al.* [1928] and explained by them as due to a diminution in the interspace water through which the diffusion was comparatively very rapid, the assumption being that there was a free passage of substance through the interspace water between the tissue elements and that these latter were not in contact. From the preceding considerations this view cannot be accepted. An explanation of the fall in the diffusion coefficient of lactate may be given as follows. If instead of the diffusion coefficient of lactic acid or anion we consider the amount diffused divided by $\sqrt{t}$, we have in effect the amount diffused in the first minute. If we plot this value against the lactate concentration in muscle we get a very striking relationship (see Fig. 4). The curve begins necessarily at

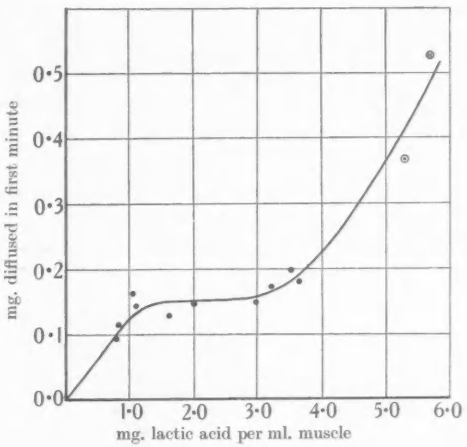

Fig. 4. This figure has been constructed from the data in the paper of Eggleton *et al.* [1928]. The dots show the concentration of lactic acid in stimulated muscle and the circles show the concentration in muscle dead at 37° (muscles R_1 and R_2 of their series).

the zero point, since with zero lactic acid in the muscle there must be zero diffusion. It then rises linearly till about 100 mg. per 100 g. lactic acid exists in the muscle. The amount diffusing then remains constant up to about 300 mg. of lactic acid. After this concentration in the stimulated muscle is reached there is a slight rise in the diffusion rate, the curve leading upwards for the high lactate values in dead muscle at 37°.

The reaching of a steady state may be explained when we consider that the cation impermeability makes the outer diffusion of anions dependent on a similar internal diffusion of anions from the external fluid. This latter must therefore impose a limitation at a certain stage. It may be possible that this

stage is reached at the beginning of the steady region of the lactic anion diffusion. At this stage it is to be noted that there also exists a large increase in free phosphate within the muscle and possibly of other diffusible anions. After 300 mg. lactic acid per 100 g. are reached in the internal concentration the cation impermeability begins, on this explanation, to break down, the process tending to completion with the dead muscles at 37°.

The passage of the anions through the cells makes the whole of the tissue-water available for solution, but owing to the operation of membrane equilibria we may get very much reduced total concentrations in the tissues compared with the external concentration. These membrane equilibria in the kidney seem to be very different from those in muscle. With the kidney in cyanide-Locke the permeation value for sulphate is 69 and with the sartorius in cyanide-Locke it is 28. However, for these two degrees of permeation, due no doubt to different degrees of membrane equilibria, the diffusion coefficients are $6{\cdot}6 \times 10^{-5}$ and $6{\cdot}3 \times 10^{-5}$ respectively—that is to say, the same within the limits of error for such determinations. The anion diffusion thus appears to be independent not only of the degree of permeation but also of the kind of tissue examined, and also it may be added of the chemical properties of the anion.

When we now consider the diffusion of urea we find that urea has a much smaller diffusion coefficient than the four anions considered. For diffusion into or out of muscle, into or out of the inactive (cyanide) kidney or out of the active kidney, the diffusion coefficient of urea is the same (within the limits of determination), namely $1{\cdot}1 \times 10^{-5}$. Such an agreement in itself, as already mentioned, disproves the conception that all the "excess" urea in the frog's kidney is in a bound condition. The permeation value of urea is 100. At first sight the low diffusion coefficient may be thought to be associated with this fact. That this cannot be so will appear when we have for lactic acid with the same permeation a diffusion rate 5–6 times faster and when the diffusion of sulphate into the inactive kidney is three times faster reckoned as a proportion of the external concentration (without a consideration of equilibrium values). The molecule of urea is also markedly smaller than three of these anions. We must therefore have a special facilitation of the passage of anions as compared with the passage of electrically neutral and even smaller and hence more rapidly moving molecules. This recalls the fact that the cations are on the other hand specially inhibited from diffusing through the cell membrane. It is possible that these are aspects of the same mechanism. It is well known that for large molecules only those which are lipoid-soluble diffuse readily into the cell, from which it is concluded that the cell membrane is mainly of a lipoid nature [*vide* Höber, 1932]. Small molecules however can readily enter the cell although they have little or no lipoid solubility. This has been explained by a pore theory, the small molecules passing through the pores in the cell membrane. It is difficult to conceive of pores except in a solid membrane and that of the cell seems to be of a fluid nature since its function persists even with marked distension or distortion. Water droplets in a membrane of such fine dimensions may, however, serve all the purposes of pores in a larger membrane. Further, if these droplets were positively charged we should have at least a working picture of the discrimination towards the passage of the differently charged ions.

Summary.

1. It has been shown, using a more correct form of the diffusion equation than that used by Eggleton *et al.* [1928], that the diffusion coefficient through the frog's sartorius of the four anions, sulphate, chloride, phosphate and lactate,

is the same within the limits of sampling error. This value is $6{\cdot}0 \times 10^{-5}$ (cm.^{2}/min.). The variation from this figure of a single estimate may be expressed as a standard deviation of $1{\cdot}3 \times 10^{-5}$.

2. This diffusion coefficient, as exemplified by sulphate, is the same through the muscle, liver and kidney of the frog, and is independent of the degree of "permeation" of the anion.

3. The equilibrium concentration of chloride in isolated muscle expressed as a ratio of the external concentration is 0·30. This compares with 0·25 for sulphate and 0·29 for phosphate (this latter being the mean value of the results of Eggleton and Semeonoff who obtained 0·21 and 0·38 respectively).

4. With the urease method employed here [Conway, 1933] in an extensive series of experiments it has been shown that the "excess" urea analysed in the frog's kidney diffuses outwards at exactly the same rate as urea diffuses inwards from free solution, and furthermore that this rate of diffusion is identical with the diffusion outwards or inwards of urea in the frog's sartorius. It is clear therefore that the urea analysed in the frog's kidney by this method is in free solution. Such urea in the freshly excised kidney of the frog gave an average value of 85 mg. per 100 g., whereas the blood value was 45 mg. and the sartorius value 41 mg. per 100 g. The diffusion coefficient of urea through muscle or kidney is $1{\cdot}1 \times 10^{-5}$.

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CCXXXV. ACCURACY IN THE DETERMINATION OF BLOOD-UREA BY THE UREASE AERATION-TITRATION METHOD.

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In recently published studies of renal function [Dunn *et al.*, 1931; Kay and Sheehan, 1933] the percentage of urea in the renal arterial blood removed from the blood during its passage through the kidney was determined. This work involved the estimation of blood-urea contents with a degree of accuracy not usually demanded; for it was essential to be able to determine with the least possible error the difference between the urea contents of blood entering and leaving the kidney at the moment of the experiment. A range of error in estimations of the urea in each sample of blood of $\pm 0{\cdot}1$ mg. per 100 ml. was permissible at ordinary blood-urea levels and a correspondingly higher range at higher blood-urea levels. Results actually within the smaller range were obtained in two-thirds of the estimations, even though the blood-urea was frequently raised to several hundred mg. per 100 ml. In this paper is given an account of the method of estimation employed: sources of error are discussed and improvements in apparatus and technique to avoid them are described.

Of the methods available, only the urease aeration-titration method of Marshall [1913], Van Slyke and Cullen [1914, 1, 2] and Van Slyke and Zacharias [1914] appeared likely to give results with a high standard of accuracy compatible with reasonably quick work; it was therefore selected. Since the original publication the method does not appear to have undergone significant changes in technique; the most recent authoritative re-statement [Peters and Van Slyke, 1932] is little more than a transcription of the first descriptions. In the latter book, an accuracy of 1 % is claimed for the method. Actually this standard does not seem to be generally attained, but by improvements in apparatus and careful attention to technique it has been found possible in the present work to obtain results with an average relative error of only $\pm 0{\cdot}2$ mg. per 100 ml. at all levels of blood-urea. These improvements deal with technical detail rather than with principle. After they had been developed it was found that a number of the points had been recognised previously by Barnett [1917] in his studies on blood-ammonia, but the implications of his work do not appear to have been applied to the method of estimating blood-urea. The technique and apparatus as finally developed have proved not only to be reliable and accurate for research but also, in our hands and others', to be easy and convenient for routine clinical work. In clinical work blood-urea can be measured on blood samples of 0·2 ml. with an average error of less than 3 mg. per 100 ml.

For the purposes of discussion, errors and improvements are grouped according as they are related to accuracy in titrimetry, to the aeration process, to the enzymic reaction or to apparatus.

Titrimetry.

The degree of accuracy aimed at was 0·01 ml. of $N/100$ NaOH in titrating 25 ml. of $N/100$ H_2SO_4. This was attained by devoting attention to a number of details of technique in measurements and titration.

1. *General.* In washing apparatus, hands *etc.* scrupulous chemical cleanliness is essential. Measuring apparatus is kept clean and free from grease by frequent cleansing with chromic acid solution. Reaction- and acid-tubes are washed separately with separate brushes, and their stoppers are never interchanged, since those of the reaction-tubes appear to absorb alkali.

2. *Type of glass.* Resistance glass must be used for all parts which are to come into contact with standard alkali or acid for more than a few minutes; monax glass is suitable.

In different soda glass tubes, the titre of 25 ml. of $N/100$ H_2SO_4 was found to fall at varying rates, usually about 0·01 ml. per hour. In monax tubes, the titre was unchanged after 24 hours.

3. *Laboratory temperature.* During measurements, the temperature of the laboratory must be kept constant, since fluctuations of 1° introduce appreciable errors. In summer the variations of temperature observed were never more than 0·2 to 0·3° during critical periods, but in winter they were sometimes so great as to prevent work of very high accuracy under ordinary laboratory conditions.

4. *Burette and pipette deliveries.* In measuring from a pipette or burette the fluid at the point is "tipped off" against the inside of a clean dry beaker before beginning the delivery. Deliveries from a burette are finished by tipping off on the bubbling tube; from an automatic pipette, by tipping off against the inner wall of the receiver to give a standard "leave" in the tip of the pipette.

5. *Automatic pipette for standard acid.* As it is difficult to maintain a high standard of accuracy with an ordinary pipette or burette, measurement of standard acid is done by an automatic 25 ml. pipette, filled from an overhead reservoir. This pipette is changed frequently to avoid errors from grease, although a moderate amount of grease in the pipette is not objectionable except in so far as it may hold air bubbles. Standard drainage time must be allowed; 2 minutes for emptying and draining were used in the present work.

Fifteen successive deliveries made from the automatic pipette used were weighed and the range of variation was found to be 0·0099 g. The deliveries were therefore constant to within 0·01 ml.

6. *The burette.* Accurate burette readings are made with a 50 ml. Schellbach blue line burette graduated in 0·1 ml. Associated conditions, *e.g.* speed of working and use of CO_2-free standard alkali solution, precluded the use of micro-burettes and weighing burettes. The burette must be kept scrupulously free from grease. All measurements are taken from the same initial point, and the portion of the burette used recalibrated by the weighing method.

Readings on such a burette can be made accurately to 0·01 ml. if certain precautions are observed. A reading tube is required to ensure that readings are made along a line perpendicular to the burette. A convenient tube is the barrel of a 5 ml. Record syringe with the entire needle mount removed; the cap mount gives a right-angled metal end, which is placed against the burette with the meniscus on the diameter. Three readings are then made with the eye at a constant distance from the burette, (*a*) looking down the centre of the tube, (*b*) looking along the inner surface of the top of the tube and (*c*) along the inner surface of the bottom of the tube. These positions are easily recognised by the

relationship of the circle of the burette end of the tube to the circle of the observer's end. In (*a*) the circles are concentric, in (*b*) the circles touch at the top, and in (*c*) they touch at the bottom. Reading (*a*) is made exactly at right angles to the burette. Readings (*b*) and (*c*) involve equal but opposite parallax, and the mean figure should be the same as that of reading (*a*). At the normal distance of reading, (*b*) is about 0·01 ml. above (*a*), and (*c*) is about 0·01 ml. below (*a*).

Measurements are most easily made by reading the short horizontal fine line of light which crosses the junction of the two points of the blue line in the meniscus, and which is enhanced by placing behind the meniscus the middle finger of the hand holding the reading tube.

Without any previous practice, it is easy to keep within an error of $\pm 0{\cdot}01$ ml. in reading the burette in this way, and the error is smaller as skill is acquired. Independent readings were made by both observers in all titrations in the present work and agreed exactly.

As a test of accuracy, eight deliveries of distilled water at 15° were made from a part of the burette which had been carefully recalibrated by weighing. Each measured volume involved two burette readings. The quantities delivered were then weighed and their volumes calculated from the known density of water. The results are given below; the greatest difference is 0·0076 ml. and the average is 0·0036 ml.

Volume by burette ml.	Volume by weighing ml.	Volume by burette ml.	Volume by weighing ml.
0·69	0·6951	0·75	0·7479
0·70	0·6966	0·76	0·7589
0·72	0·7232	0·78	0·7724
0·74	0·7460	0·81	0·8100

7. *Indicators.* The indicator usually employed for titrations was methyl red. The critical colour change selected as the end-point occurs after a gradual change from red to reddish yellow; there is then an abrupt change to greenish yellow accompanied by an increase of brightness due to discharge of the red shade. Using CO_2-free solutions this change is brought about in 40–50 ml. of fluid by less than 0·01 ml. $N/100$ NaOH in the absence of ammonium salts, and by no more than 0·01 ml. $N/100$ NaOH even when ammonium salts are present, the colour change being instantaneous and permanent so long as a CO_2-free atmosphere is preserved. In this volume of fluid the amounts of indicator required for satisfactory recognition of the colour change are 0·2 ml. of 0·02 % methyl red in 50 % alcohol, or 0·3 ml. of the B.D.H. stock solution. These amounts must not only be fixed but must also be accurately measured. If a micro-burette is used, the tip must be enclosed between measurements to prevent evaporation of alcohol and the formation of crusts of indicator. Accurate measurement is necessary because each 0·1 ml. of the indicator solution used requires 0·02 ml. of $N/100$ NaOH to produce the colour change.

Titration of acid which contains drops of octyl alcohol is exceedingly difficult, as some of the methyl red is dissolved in the alcohol giving an orange solution which does not take part in the colour change. This obscures the critical colour change. The present aeration method dispenses with the use of the anti-foam liquid in the acid and this complication does not arise.

Another satisfactory indicator is the "4·5" indicator of B.D.H. This has the advantage of having a colour change after the critical one used as the end-point; there is thus less danger of not recognising over-titration. Near the

end-point abrupt colour changes occur with each addition of 0·01 ml. $N/100$ NaOH to 50 ml. of solution being titrated, these are (*a*) grey to (*b*) dull green-blue to (*c*) a clear blue; the addition of a further 0·01 ml. $N/100$ NaOH causes a marked change to an intense bright blue which is distinctive.

The critical colour changes in these two indicators can only be recognised if the titration is completed by the addition of separate amounts of 0·01 ml. of the $N/100$ NaOH. It is advisable for the observer before using any indicator to carry out preliminary test titrations to familiarise himself with the critical colour change.

8. *The avoidance of CO_2.* The avoidance of CO_2 in the solutions involved in titration is one of the principal factors in securing accuracy; the effects of CO_2 in titrations involving $N/100$ solutions are generally underestimated [see also Acree and Brunell, 1906; Marshall, 1913]. A series of experiments was conducted to investigate this point quantitatively. 25 ml. of $N/100$ H_2SO_4 were titrated to near the end-point with $N/100$ NaOH. Small measured quantities of $N/100$ Na_2CO_3 were then added and the titration completed with the $N/100$ NaOH (to avoid removal of the added CO_2, the solution was not aerated during the final titration). Each addition of 0·2 ml. $N/100$ Na_2CO_3 in these titrations corresponds to 1 % Na_2CO_3 in the total solid alkali present. The results are shown in the table below. The essential points are that with increasing amounts of carbonate present:

(*a*) Steadily increasing quantities of total alkali are required.

(*b*) The end-point becomes spread more widely, *i.e.* the amount of alkali required to complete the critical colour change is increased.

(*c*) The colour change is harder to recognise owing to the bleaching of the indicator. This is marked when 0·4 ml. of $N/100$ Na_2CO_3 is present, and makes titration very difficult when 0·8 ml. is present.

(*d*) "Swing back," *i.e.* return to the previous colour, occurs owing to the buffering action of the carbonate so that more alkali must be used to produce permanence of the end-point.

$N/100$ Na_2CO_3 added ml.	Total alkali used in titration $N/100$ NaOH + $N/100$ Na_2CO_3 ml.	End-point	
		"Spread" $N/100$ NaOH ml.	Swing back $N/100$ NaOH ml.
0	25·71	0·01	0
0·1	25·75	0·02	0·01
0·2	25·76	0·04	0·02
0·3	25·78	0·04	0·03
0·4	25·81	0·06	0·04
0·5	25·83	0·07	0·05
0·8	25·94	0·08	0·05

In the titrations the most important source of CO_2 is the standard alkali. The avoidance of this CO_2 is most fundamental and important. The method of preparing CO_2-free standard NaOH solutions and the fitting of a burette for their use is described in a subsequent paper [Kay and Sheehan, 1934].

It is also very important that the air coming into contact with the standard acid during titrations should not contain CO_2. This is secured by passing a stream of air washed free from CO_2 and NH_3 through the acid during titration. A convenient source of clean air is obtained by passing the outflowing frothy water from a water suction-pump into a large Woulff's bottle. The water is siphoned away from the bottom and the liberated air is led from the upper part of the bottle through a stop tap and then through caustic soda and

sulphuric acid wash-bottles and finally through distilled water and an empty guard-tube to the acid.

Distilled water used for preparing standard acid or alkali is boiled thoroughly in a flask before use and cooled out of contact with ordinary air by connecting up to a caustic soda wash-bottle.

9. *Technique of titration.* The method of titration is as follows.

After use, the burette is always left full of alkali. As this alters in strength on standing even for half an hour in an ordinary burette, it is changed completely before starting titrations.

After filling the burette and adjusting to zero, a drainage time of 1 minute is allowed before reading. Meanwhile the exact amount of indicator is added to the acid-tube. The stopper of the acid-tube is raised but not removed and the bubbling tube is then connected by rubber tubing to the stream of air freed from NH_3 and CO_2; this stirs the liquid efficiently during titration and preserves a neutral atmosphere.

Titration is then proceeded with, timed by stop-watch, until the end-point is approached and about 0·2 ml. of alkali has still to be added. The acid-tube is then removed from the burette, stoppered, disconnected from the air stream and shaken twice to wash down the walls. The acid remaining in the bubbling tube is blown out twice into the rest of the solution by temporary connections of the air stream tubing, and a proper connection is finally made again. All the acid in the tube is thus collected for the complete titration. To obtain an accurate end-point the titration is completed by "tipping off" portions of a drop of alkali (about 0·01 ml. each) from the burette tip on to the bubbling tube and transferring them to the acid.

The burette is read exactly 3 minutes after starting to run in the alkali.

As a test of the reliability of measurements and titration, five successive lots of $N/100$ H_2SO_4 were measured by the automatic pipette and titrated by the standard method. In each case the amount of standard alkali required was exactly 21·91 ml.

The aeration process.

1. *Rate of aeration.* The rapid short aeration (5 litres of air per minute) recommended by previous workers was not used in view of the alkaline spray which must inevitably have interfered with accuracy in their form of apparatus. The rate used was 1 litre of air per minute for 2 hours, standardised by the timed displacement of a measured volume of water. The total volume of air passed is roughly the same as that claimed by other workers to be enough; ammonia transport depends on the volume of air passed rather than on any factor of time or rate [Van Slyke and Cullen, 1916].

The quantitative recovery of ammonia by aeration claimed by other workers [Folin and Macallum, 1912; Kober and Graves, 1913; Van Slyke and Cullen, 1916; Folin and Wu, 1919] cannot be accepted; it is probably due to inadequate precautions to control alkaline spray which thus reaches the $N/100$ acid and is estimated as ammonia, so that sometimes more than 100 % of the actual ammonia is accounted for. The present work has been done with almost complete avoidance of alkaline spray, the performance of adequate blanks to account for such constant traces of spray as do pass and very accurate titrations. With four quantities of pure NH_4Cl varying from 0·669 to 10·698 mg. and the passage of 120 litres of air in 2 hours, only about 96 % transfer of NH_3 was found. Greater transfer can be obtained by more prolonged aeration at the same rate but complete transfer does not occur except possibly with very small

quantities of ammonia. This is shown from the figures below for two different amounts.

Duration of aeration hours	Percentage of NH_3 transported from	
	0·669 mg. NH_4Cl	10·698 mg. NH_4Cl
½	79	92·1
2	96	95·6
2½	97	96·1
4	97	96·3
12	98	97·2
23	100	97·8

Heating the reaction tubes to 50° appears to accelerate the transfer of NH_3, but the method was not applied to blood in view of the strong alkali present.

The addition of urease to the NH_4Cl has no obvious effect on the efficiency of aeration.

Though the 2 hours' aeration does not transport all the ammonia it was proved in many experiments that all tubes aerated together in series gave the same percentage transport when the amounts of NH_3 were not widely different from each other.

2. *Alkaline spray.* As mentioned above, there is a great danger of carrying over alkaline spray during aeration from the reaction-tubes to the acid-tubes. Various workers have dealt with this problem by putting cotton-wool plugs in the outlet tubes of the reaction-tubes [*e.g.* Barnett, 1917]. Such plugs are relatively efficient; the chief objections to their use, apart from possible absorption of traces of NH_3, are the interference with smooth aeration in serially connected units and the necessity for strong suction to overcome their resistance.

Very satisfactory control of alkaline spray was obtained in the present work by using a zig-zag resistance glass connecting tube between the reaction- and acid-tubes. This is bent at 15 points; 11 of these are in the ascending limb as this arrangement was found most effective. During aeration, alkali is deposited up to the 11th bend where the tube finally becomes horizontal, but none can be shown in the long straight descending limb by subsequently running indicator along the tube. The minute amount of alkaline mist which passes through the tube to the acid is proportional to the rate as well as the time of aeration, but is quite constant for all tubes aerated in series and is therefore accounted for in the blank. At the standard rate of aeration it neutralises slightly less than 0·01 ml. of $N/100$ H_2SO_4 per hour, corresponding to about 0·0000086 ml. of the strong alkaline solution in the reaction-tube.

These glass connecting tubes are joined to the tubes from the reaction- and acid-tubes by short pieces of rubber tubing lubricated with a little distilled water, and the ends of the glass tubes are pushed together so that the air stream does not come into contact with the rubber tubing.

Barnett [1917] and Folin and Wu [1919] have drawn attention to the chemical defects of the long pieces of rubber tubing usually employed for connecting the reaction-tubes to the acid-tubes. They are certainly not satisfactory for accurate estimations; in the present work the average difference between 16 pairs of estimations of urea in a series using rubber tubing connections was over four times greater than in the series using the glass connections, the rest of the technique and apparatus being the same.

Soda glass connecting tubes are theoretically not quite so satisfactory as resistance glass, owing to the danger of moisture condensing in the downward limb and falling with alkali dissolved from the glass into the acid. This is not

however of great significance; the results were very nearly as good in 62 pairs of estimations of urea using soda glass connections as in the present larger series using resistance glass.

3. *The addition of potassium carbonate.* Many errors arise from the usual addition of solid potassium carbonate to liberate ammonia. The reaction-tube has to be opened; this involves possible loss of ammonia. As there is usually gross spilling, the amount of potassium carbonate added is never constant, and as the carbonate often contains traces of ammonia it is not possible to do a satisfactory blank. This is however not of such importance as the fine cloud of K_2CO_3 dust which remains for some time in the upper part of the reaction-tube and some of which may be carried by aeration into the $N/100$ acid. Only 0·000007 g. of solid K_2CO_3 is required to neutralise 0·01 ml. of $N/100$ acid: very small traces may therefore produce serious errors.

All these sources of inaccuracy are avoided by the use of saturated solution of K_2CO_3 as suggested by Fiske [1915]. The statement of Van Slyke and Cullen [1916] that the difference between the use of K_2CO_3 as a solid and in solution is "one of convenience rather than accuracy" cannot be accepted. The amount of saturated K_2CO_3 solution required for blood-urea estimations is that to produce two-thirds saturation in the final mixture. Greater amounts produce no improvement; half-saturation as recommended by Van Slyke and Cullen is almost but not quite as good. The measured 20 ml. of saturated K_2CO_3 solution required for the usual volume of fluid in the reaction tube are added by a tap-funnel, which is an integral fitting in the reaction-tube stopper. This is clean and accurate in use and avoids the danger of ammonia loss. The alkali should be added drop by drop during aeration, taking 3–5 minutes, in order to avoid too coarse precipitation of the blood-proteins. The last few drops must not be allowed to blow spray into the reaction-tube.

The presence of NH_3 in the K_2CO_3 solution must be tested for with Nessler's solution. If NH_3 is found in substantial amount it must be removed before using the solution by prolonged aeration with clean air; any traces remaining are dealt with by the blank.

4. *The prevention of frothing; size of reaction-tubes.* The reaction-tubes (preferably of monax glass) should be at least 250 mm. long × 30 mm. diameter to allow satisfactory aeration[1]; this is much larger than the tubes which are usually used. To prevent frothing in the alkaline blood-containing liquid, technical *iso*butyl alcohol was preferred to octyl alcohol as some samples of the latter are not efficient for the purpose. In addition, the portion of the *iso*butyl alcohol that volatilises and is thus transported to the acid is completely dissolved in it, whereas octyl alcohol tends to form droplets which interfere with titration.

2 ml. of *iso*butyl alcohol were added through the tap-funnel of each reaction-tube after incubation; the tubes must be cooled for 5 minutes in cold water in the incubating bath before this addition in order to avoid distillation of the alcohol into the glass connecting tubes.

5. *The acid-tubes.* The acid-tube is the same size as the reaction-tube and is sufficiently big not to allow frothing to any dangerous height; it is therefore not necessary to use anti-foam liquid in the acid. The importance of this in the subsequent titration has already been pointed out, but the great advantage in the present connection is that a layer of bubbles about 1–2 cm. deep can be maintained on the surface of the acid throughout the aeration, thus prolonging the contact of air with acid and increasing the area of contact. This and the relatively slow rate of aeration give complete absorption of NH_3 by the acid;

[1] Recently, reaction- and acid-tubes 300 × 34 mm. have been used.

in tests, a second acid-tube after the first remained unaffected in titre. It is thus permissible to connect up estimating units in series; the actual results of such serial aerations never gave any indication of passage of ammonia from one unit to the next.

6. *Purification of air for aeration.* The air used for aeration is passed first through 20 % NaOH, then through 25 % H_2SO_4 and distilled water. The acid-tube must not precede the alkali-tube because any spray carried out of the acid-tube is liable to contain arrested ammonia. The efficiency of the NH_3 trap is such that, in a test, 6 hours' aeration through $N/100$ H_2SO_4 did not produce any measurable effect on the titre of the acid.

Hydrolysis of urea.

1. *The urease solution.* The urease solution was made by extracting freshly ground soya bean meal in 10 times its weight of distilled water for 1 hour. To obtain unvarying blank values the solutions must be quite homogeneous; this is attained by thorough centrifuging after straining through gauze. The ammonia which occurs in this urease solution is simply and exactly accounted for by the "blank" estimation done with every batch of estimations. It is possible to remove this NH_3 [Folin and Youngburg, 1919; Van Slyke and Cullen, 1914, 2], but it does not appear necessary. 5 ml. of the urease solution are used with 5 ml. of 0·6 % KH_2PO_4 buffer and incubation conducted for 30 minutes at 50°.

2. *The efficiency of hydrolysis.* In view of the incompleteness of aeration transfer of NH_3, 95–96 % estimation of urea is to be considered as indicating practically complete hydrolysis. Though hydrolysis of pure urea is not always complete, especially with small amounts, the proportion of any given quantity is always the same in solutions incubated and aerated under the same conditions. Thus, for example, eight paired estimations of pure urea solutions gave an average titration difference between pairs of 0·01 ml. $N/100$ NaOH, which is within the error of measurement.

Working with blood, the hydrolysis appears to be very constant and complete for varying amounts of urea and of blood. The following figures were obtained by paired estimations of different quantities of a sample of blood.

Volume of blood ml.	mg. urea per 100 ml. blood	
3	32·1	32·0
2	32·2	32·1
1	32·2	32·0
0·2	32·0	33·0

Hydrolysis of urea added to blood appears to be complete. Various quantities of urea were added to 2 ml. of blood whose urea content had been found to be 0·848 mg. The estimations gave results as follows, the slightly higher figures with small amounts of urea being accounted for by the relatively greater efficiency of aeration with small amounts of NH_3:

Amount of urea added (mg.)	Percentage of total urea estimated
0·625	96·0
1·250	95·6
5·000	95·0

With aqueous solutions of pure urea on the other hand, hydrolysis is incomplete when small quantities of urea are used though it is complete when large quantities are used. This is shown below in a series of estimations.

Actual urea mg.	Percentage estimated	Actual urea mg.	Percentage estimated
0·382	81·0	1·528	94·2
0·625	88·0	3·057	94·8
0·764	87·0	5·000	94·9
1·250	94·1	6·113	95·1

The use of soya bean extract obtained by extracting 20 g. of meal with 100 ml. of water gives more complete hydrolysis of small amounts of urea in water than those shown above. Thus with 0·382 mg. urea 96 % was estimated, and with 6·113 mg. 96·2 %. The use of this strong extract is not however necessary for estimations on blood.

More prolonged incubation also gives more complete hydrolysis; after incubation of 0·382 mg. urea for 2 hours with 10 % soya bean extract, 94 % was estimated. This long incubation is without effect on the soya bean extract blank but is to be avoided in work with blood as it allows the possibility of urea formation from interaction of blood-arginase with the arginine in the soya bean extract [Behre, 1923; Addis, 1928].

The conditions governing the complete hydrolysis of urea have been further investigated and an account is given in a subsequent paper [Kay and Reid, 1934]. The results of that work indicate that urea in blood undergoes practically complete hydrolysis and explain the relative inefficiency of hydrolysis in watery solutions of pure urea.

Apparatus.

1. *Blood pipettes.* Blood pipettes are calibrated to contain an exact volume. Errors up to ±2 % often occur in makers' graduations. Recalibration by the mercury weighing method can be made to an accuracy of ±0·1 %. To allow easy washing out of blood in delivery the bore should be about 1·0 to 1·5 mm., slightly narrower at the tip, the bulb fusiform, the top funnel-shaped to take the tip of the phosphate buffer pipette, and the tip ground to 60° so as to have as little glass at the point as possible.

2. *The blood-urea apparatus*[1]. The principal changes have already been indicated. Full details can be seen in Fig. 1. The apparatus is designed not to require opening from the first connecting-up until the end of the aeration, in order to avoid loss of NH_3. First the acid-tubes are charged and stoppered; next the reaction-tubes are charged with blood, buffer and urease, and stoppered. The reaction- and acid-tubes of each unit are connected by the zig-zag glass connecting tube previously described and the units are connected serially by rubber tubing. The battery of units is supported rigidly in a metal stand which is saddle-shaped (see Fig. 2), so that the reaction-tubes assembled on one side can be placed in a water-bath and the acid-tubes on the other side left outside. After assembling, the battery in its stand is an entity which can be incubated and subsequently lifted out on to the bench without disturbing the units. After the cooling, the *iso*butyl alcohol and K_2CO_3 solution are added through the tap-funnels during aeration.

In assembling the apparatus distilled water in minimum quantity is used as lubricant for rubber-to-glass joints. The first pair of tubes to receive incoming air during aeration must be that for the blank estimation. This avoids a slight error which may arise from blood being forced up the bubbling tube of the first unit during incubation and so partly escaping heating, and later, alkali. During incubation the reaction mixture is stirred from time to time by gently

[1] The improved blood-urea apparatus is supplied to the specification of this paper by Messrs Griffin and Tatlock, Ltd., 34 Great Ducie St, Manchester.

drawing a few bubbles of air through it. This allows for expansion of air and equalises the pressure inside the apparatus, thus preventing blood from being forced up the bubbling tubes.

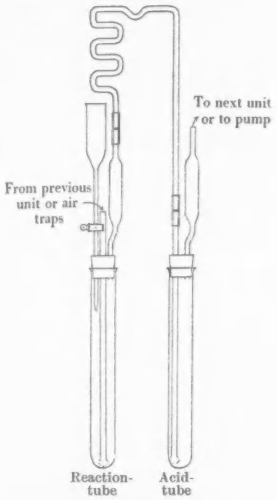

Fig. 1. Blood-urea apparatus. ($\times \frac{1}{3}$.)

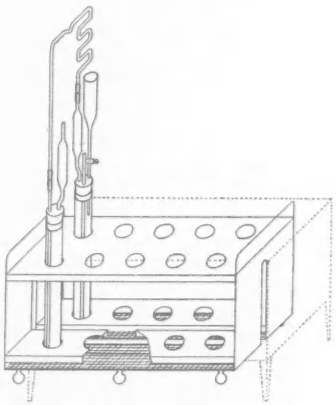

Fig. 2. Saddle-shaped metal stand for blood-urea apparatus. ($\times 1\frac{1}{2}$.)

Note on potassium oxalate. Some samples of potassium oxalate (used to prevent clotting of blood) contain NH_3. They must be tested with Nessler's solution; only those ammonia-free should be used.

Accuracy of the method.

Errors of measurement and titration are reduced to very fine limits, the urease hydrolysis in blood appears to be almost quantitative, but the aeration transfer of NH_3 is not complete. As a result the method gives with blood an absolute accuracy of only about 96 %, but the relative accuracy is limited only by that of the measurements. The high standard of relative accuracy is satisfactory from the point of view of the renal function studies for which the method was developed.

The test of relative accuracy is to examine the differences in terms of ml. of standard alkali (*N*/75 NaOH) between the titres of duplicate estimations on the same blood carried out simultaneously under identical conditions. Ninety consecutive pairs of estimations of blood-urea by the present method are available, mostly on 2 ml., the rest on 1 ml. The bloods had urea contents ranging from 14 to 600 mg. per 100 ml., the average being 93 mg. Magnitudes of differences are independent of blood-urea level. The distribution of differences is as follows:

Difference between pairs ml. of *N*/75 NaOH	Number of pairs		Difference between pairs ml. of *N*/75 NaOH	Number of pairs
0·00	17	80 %	0·05	1
0·01	42		0·06	3
0·02	13		0·07	1
0·03	4		0·09	1
0·04	7		(0·21 !)	1

In any pair of estimations at normal blood-urea levels the range of possible error due to measurements is as follows:

	ml. $N/75$ NaOH
Blood pipette measurements	0·003
Automatic pipette measurements	0·010
Burette measurements	0·015

The total possible error of measurement is thus 0·028 ml. $N/75$ NaOH, though at high blood-urea levels the error due to blood pipette measurements is proportionately higher. Actually the mean of the differences in the 90 pairs of estimations is 0·02 ml. $N/75$ NaOH, and this average includes one quite anomalous pair where some gross accidental error must have occurred. 80 % of the pairs have a difference of 0·02 ml. or less; *i.e.* within the average.

Using 2 ml. of blood for each estimation this difference of 0·02 ml. $N/75$ NaOH corresponds to 0·4 mg. urea per 100 ml. blood, on an average blood-urea in the present experiments of 93 mg. per 100 ml.

Summary.

1. Sources of error in the urease aeration-titration method of estimating blood-urea are discussed, and improvements in technique and apparatus to overcome them are described.

2. The aeration method only removes about 96 % of ammonia under the conditions described so that the method only estimates this proportion of urea present.

3. Nevertheless the modified method gives a very high standard of relative accuracy for all levels of blood-urea. In estimations of blood-urea ranging from 14 to 600 mg. per 100 ml. the average difference between pairs corresponded to 0·02 ml. of $N/75$ alkali, or 0·4 mg. urea per 100 ml. of blood.

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CCXXXVI. THE PREPARATION, STORAGE AND USE OF STANDARD CARBONATE-FREE SODIUM HYDROXIDE SOLUTIONS.

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When adapting the urease aeration-titration method for very accurate determinations of blood-ureas [Kay and Sheehan, 1934] it became evident that the primary essential for accuracy was that the $N/75$ or $N/100$ NaOH used for titrations should be absolutely free from carbonate. Dilution of strong or saturated solutions of NaOH failed to give good enough solutions; the method described in this paper was therefore devised, and has given complete satisfaction throughout a long period. It appears to be a new application of a well-known simple chemical reaction, though Sutton [1904], to precipitate carbonates from caustic soda solutions, recommended the cautious addition of baryta.

Barium hydroxide is allowed to react with excess of carbonate-free sodium sulphate in aqueous solution: after settling, a solution of carbonate-free NaOH containing sodium sulphate is obtained.

$$Ba(OH)_2 + Na_2SO_4 \rightarrow BaSO_4 + 2NaOH.$$

The presence of Na_2SO_4 in the alkali does not interfere with titrations.

The possible significant impurities in reagents are chlorides and carbonates in the sodium sulphate, barium carbonate in the baryta, and CO_2 in the distilled water. The chloride plays no significant part in the reaction, as any barium chloride which it may form gives barium sulphate, so that all barium is precipitated. Carbonate is removed from the sodium sulphate by making the solution very faintly acid with H_2SO_4 and boiling vigorously before adding the baryta. CO_2 is removed from distilled water by boiling. The slight traces of barium carbonate suspended in the baryta solution might theoretically cause difficulty but do not in practice.

Preparation of concentrated alkali.

The method is carried out as follows:

A flat-bottomed litre flask (monax or other resistance glass) is fitted with a tight rubber stopper through which pass

(*a*) a short bent glass tube connected to two wash-bottles in series, each containing 40 % NaOH. These serve as CO_2 traps for air entering the flask;

(*b*) one limb of a long glass siphon preferably of resistance glass. This is a fairly tight fit in the stopper but can be forced up or down as required; in the preliminary stages it is pulled up so that it only projects a short distance below the stopper. The other limb of the siphon is sealed into a good glass tap, the delivery tube of which is about 5 cm. long, drawn out into a jet about 2 mm. bore, and protected from air by being enclosed in a small removable test-tube.

Details can be seen in Fig. 1.

800 ml. of distilled water and 300 g. of pure sodium sulphate crystals are placed in the open flask and boiled vigorously for 10–15 minutes. The solution is made faintly acid with dilute H_2SO_4 (usually 1–2 ml. N H_2SO_4 are ample) and boiling is continued for several minutes.

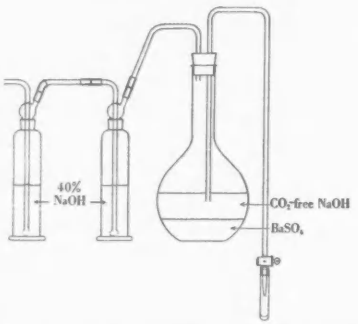

Fig. 1. CO_2-free NaOH solutions. Preparation and storage apparatus.

Meanwhile in another flask a solution is made of 280 g. of pure barium hydroxide crystals in 200 ml. of distilled water, and boiled vigorously. There is usually a slight precipitate of barium carbonate.

The boiling baryta solution is added to the boiling sulphate solution. There is an immediate precipitation of barium sulphate, which causes a tendency to boil over. When all the baryta is added, boiling is continued for a few minutes to help granulation of the precipitate. The flask is then stoppered, thus connecting it to the CO_2 traps, removed immediately from the source of heat and allowed to settle and cool. The siphon tap must of course be closed and, to avoid fouling of the siphon with precipitate, the uptake limb should not yet enter the solution.

The barium sulphate settles rapidly and leaves a perfectly clear solution of carbonate-free NaOH, about 1·2–1·3N and containing Na_2SO_4. The solution is ready for standardisation and use as soon as clear and cold. The uptake limb of the siphon is then pushed down into the solution and the siphon started either by suction at the jet or by blowing gently through the CO_2 traps.

Prepared and stored in this way, carbonate-free NaOH keeps perfectly for 4–5 months. Solid carbonate should not be allowed to form round tap or jet (the tube enclosing the jet prevents this) and the jet is cleaned and dried externally before and after use. The solution in the tap and siphon is rejected when withdrawing solution for use.

Preparation of dilute alkali.

For the preparation of $N/100$ NaOH, the calculated amount of concentrated solution is drawn into a narrow measuring cylinder and added at once to the requisite amount of boiling distilled water in a flat-bottomed resistance glass flask, the water having been boiled vigorously for some time to expel CO_2. The flask is then stoppered and removed from the source of heat, the stopper having a connection with a wash-bottle containing 40 % NaOH, through which air is admitted to the flask in cooling.

When the solution is cold, the flask is connected to a burette fitted up as in Fig. 2. The flask is supported on a movable shelf. The solution is then standardised for use.

Attention may be called to the following points:

(*a*) The burette is filled from the top; this eliminates inaccuracies through faulty 3-way taps, and grease is not carried up the burette from the tap. The tap through which the burette is filled hardly ever needs greasing. The jet which discharges the solution into the burette is bent slightly to direct the stream against the burette side in order to avoid bubble formation.

(*b*) The small amount of air making contact with the solution is never greater than the volume of the solution used. It passes through two wash-bottles containing 40 % NaOH solution, followed by one containing distilled water, when entering to replace solution run out of the burette; when the burette is filled again the air from it is displaced into the reservoir. If all the joints are satisfactorily air-tight the burette cannot be emptied completely owing to the head of fluid in the CO_2 traps. This is not important as only the upper half of the burette need be used for titrations.

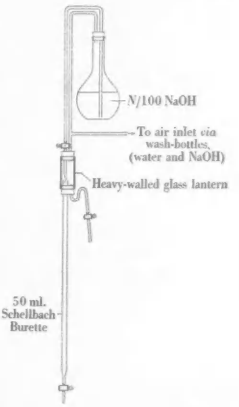

Fig. 2. CO_2-free NaOH solutions. Ordinary burette fitted for use with diluted standard solutions. The flask serves as dilution flask and as reservoir for the diluted solution.

(*c*) The standard solution never comes into contact with rubber.

(*d*) The glass of ordinary burettes has a deleterious effect on *N*/100 NaOH, the strength of which is changed by as much as 0·1–0·2 % on standing for 1–2 hours in the burette. Solutions left in the burette therefore must be rejected and thoroughly changed before use.

On the other hand, under the conditions described, dilute alkali can be stored in monax vessels for at least 10 days without change of strength or loss of sharpness of end-point. The critical colour change of methyl red in 40–50 ml. of fluid is produced by less than 0·01 ml. *N*/75 NaOH [see Kay and Sheehan, 1934].

(*e*) When placing a new dilute alkali solution in position above the burette, the ascending limb of the siphon is wiped dry with clean cotton-wool and a little solution run out of the siphon to break contact between old and new solutions.

Summary.

1. Very satisfactory carbonate-free NaOH solutions can be prepared by the interaction of barium hydroxide and pure sodium sulphate.

2. A description is given of the technique of preparing and storing such solutions and of their dilution and use in a burette.

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CCXXXVII. THE OPTIMUM BUFFER p_H FOR HYDROLYSIS OF UREA BY UREASE, AND THE PREPARATION OF STABLE UREASE POWDER.

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When estimating urea by their improved technique Kay and Sheehan [1934] found that hydrolysis of urea in aqueous solution was not always complete, especially with small amounts, whereas under identical conditions hydrolysis of urea in blood appeared always to be complete; stronger extracts of soya beans also gave more nearly complete hydrolysis. Both blood and soya bean extracts are slightly alkaline. It appears probable therefore that the 0·6 % KH_2PO_4 solution used as buffer is too acid in reaction for complete hydrolysis of urea in aqueous solution. When this buffer is partly neutralised by adding a quarter of its equivalent quantity of NaOH, estimations show almost complete hydrolysis of even small amounts (*e.g.* 0·5 mg.) of urea in aqueous solution. These facts suggest that a systematic investigation of the effect of the buffer used would be of value, especially as a technique capable of high accuracy is now available. For this purpose a stable urease preparation is needed. Attempts to prepare crystalline urease by Sumner's method [1926] failed.

In the present paper we give an account of the preparation of a dry, stable urease powder, and of an investigation of the effects of different phosphate buffers on the efficiency of the hydrolysis of urea in aqueous solution by this urease, with a view to defining the optimum buffer p_H.

The preparation of a stable urease powder.

Finely ground and sifted jack bean meal was digested at room temperature for one hour with five times its weight of distilled water. Then by straining through fine cotton gauze and thoroughly centrifuging the liquid, a homogeneous extract was obtained. This was concentrated to a syrupy consistency under reduced pressure at 25–29° with gentle agitation by a fine, slow stream of CO_2 bubbles, which also prevented frothing and bumping. 140 ml. of concentrate (from 500 ml. of original extract) were poured into 500 ml. of acetone, with brisk stirring, the resulting precipitate being filtered off on a suction-filter and washed with acetone.

One portion of the sticky, rubbery mass obtained was dried *in vacuo* over concentrated sulphuric acid at room temperature, and yielded a hard, horny mass which was powdered. Another portion was ground with pure dry acetone and became a coarse, granular powder, which, after filtering, washing with dry acetone and drying in air at room temperature, was easily ground to a fine powder. The second method of treatment is preferable as it is quicker and less

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laborious. Subsequent tests of activity showed that the two powders were of almost identical strengths; they were therefore mixed and became the stock source of urease. In all, 47 g. of dry powder were obtained from 1000 ml. of original aqueous extract.

The final powder is slightly yellowish in colour and dissolves readily in water or phosphate buffer solutions to give an opalescent solution. It has a faintly acid reaction (0·02 % solution has p_H 6·5–6·6) and exercises only a slight buffering effect. It is so nearly free from ammonia that "blank" estimations in the aeration-titration method of estimating urea represent alkaline spray only. It has shown no evidence of deterioration after 10 months in the laboratory without special storage precautions.

Tests of activity of the urease powder.

1. *Comparison with original jack beans.* Different amounts of urease powder in aqueous solution were incubated for 10 minutes at 50° with 5 ml. of 3 % urea solution in phosphate buffer of p_H 6·4. The reaction was stopped by addition of mercuric chloride and the ammonia formed titrated. The same procedure was carried out with different volumes of a jack bean extract, prepared as described above.

Equivalent hydrolysis of urea was given by 50 mg. of urease powder and by 5 ml. of jack bean extract (equivalent to 1·0 g. of meal), which hydrolysed 138 and 139 mg. of urea respectively. The activity of the powder is thus about twenty times that of the meal. Since the yield of powder is rather less than 25 % of the weight of meal it thus appears as if the isolation process not only concentrates the urease but actually increases its hydrolytic power.

2. *Hydrolytic power of the stock urease powder.* Using phosphate buffers of different p_H values, 0·5 mg. of stock urease powder was allowed to act in this case for 30 minutes at 50° on 150 mg. of urea. The reaction was stopped by adding K_2CO_3 and the ammonia formed estimated by aeration and titration. The following results were obtained:

At p_H	mg. urea hydrolysed	At p_H	mg. urea hydrolysed
5·8	3·4	6·8	4·7
6·0	4·3	7·0	3·1
6·4	4·7	7·2	2·7

Under the most favourable conditions of p_H, the powder hydrolyses more than nine times its own weight of urea, whilst even under the most adverse conditions, at p_H 7·2, it hydrolyses at least five times its own weight of urea. This is stronger than the powder prepared by Van Slyke and Cullen's method [1914] but much weaker than Sumner's recrystallised urease, which had about 1000 times the activity of the jack beans from which it was prepared [Sumner and Hand, 1928].

It was considered therefore that in subsequent estimations 2 mg. of urease powder would be a safe excess for the quantities of urea used (0·5–6 mg.). Results proved this to be so.

The effect of buffer p_H on the efficiency of urea hydrolysis.

Known amounts of pure urea, varying from 0·5 to 6·0 mg. were estimated in duplicate, using 2 mg. of stock urease powder and phosphate buffers varying in p_H from 4·5 to 8·4. The technique and apparatus were exactly as described by Kay and Sheehan [1934] except that a larger volume of air was used for aeration.

Pure urea was prepared by recrystallising dry, chemically pure urea from pure dry acetone and finally drying *in vacuo* over concentrated H_2SO_4 for 12–16 hours before use. Solutions of appropriate strength in distilled water were made as required, so that the desired amount of urea could be exactly measured in accurately calibrated 1 or 2 ml. blood pipettes.

The phosphate buffers were made by mixing $M/5$ solutions of KH_2PO_4 and NaOH according to the standard tables of Clark [1928]. 5 ml. were used in each estimation.

A 1 % stock solution of urease powder in p_H 7·0 phosphate buffer was made. This keeps under ordinary laboratory conditions for at least a week, but actually was renewed at shorter intervals. It was diluted as required by appropriate buffers so that the resulting solutions contained 2 mg. of powder in 5 ml., the volume used in the estimations. The p_H of the buffer solutions was not appreciably affected by the urease.

The total volume of urea-urease-buffer mixture in each estimation was always 12 ml., 1 ml. of distilled water being added when only 1 ml. of urea solution was used.

The results are set out in Table I, the averages of the paired estimations being given. The agreement between members of each pair compared favourably with that obtained by Kay and Sheehan [1934], the average difference between members corresponding to 0·022 ml. $N/75$ NaOH. In Table I account is not taken of the small portion of ammonia which aeration fails to transport, but as this is almost constant the figures are comparable amongst themselves. Owing to the larger volume of air used in aeration as compared with that employed by Kay and Sheehan, in the present work values of 98–99 % may be taken to represent complete hydrolysis. Tests of the alkaline liquid after aeration constantly showed small amounts of untransported ammonia.

Table I. *Efficiency of hydrolysis of different amounts of urea at varying buffer* p_H *values.*

Urea used mg.	0·5	1·0	1·5	2·0	4·0	6·0
	Percentage of urea estimated					
p_H of buffer						
4·5	7·0	9·8	12·2	14·8	54·9	78·1
4·7	15·6	17·6	28·1	35·5	75·7	93·8
5·8	58·0	66·6	78·5	85·3	91·3	94·6
6·2	94·5	94·3	97·0	97·9	94·4	93·9
6·3	—	—	—	—	97·7	98·1
6·4	93·9	95·8	98·4	98·7	98·5	98·0
6·6	96·8	97·9	99·0	97·8	98·3	98·9
6·8	96·5	97·7	99·0	98·7	98·0	98·2
7·0	96·1	97·1.	98·4	98·7	98·6	99·2
7·2	95·8	98·2	99·0	98·6	98·1	98·9
7·6	—	—	—	—	98·3	98·3
7·8	96·6	97·9	98·7	98·8	96·9	94·3
8·0	—	—	—	—	97·0	87·4
8·4	94·2	94·4	97·1	96·3	71·5	71·0

The poor efficiency with buffers of low p_H is apparently due to the medium being too acid. On the other hand, the fall in efficiency with large amounts of urea and buffers of high p_H is to be attributed to the medium becoming too alkaline for urease to work satisfactorily. Van Slyke and Zacharias [1914] said that although the neutral point is the optimum p_H, with decreasing amounts of urea the p_H should increase to obtain optimum conditions.

Evidently for general purposes, for estimating urea in aqueous solutions or in blood, an initial reaction of p_H 6·6–7·0 is most satisfactory. This range is given by KH_2PO_4 solution to which one-third to one-half of its equivalent of NaOH is added, the final solution being adjusted to represent a concentration of 6 g. per litre of original KH_2PO_4. Thus to 6 g. KH_2PO_4 dissolved in a little distilled water add 0·6–0·88 g. NaOH and make up to 1 litre. This buffer is very similar to that recommended by Peters and Van Slyke [1932]. The optimum buffer p_H values and the relative efficiency of hydrolysis for different amounts of urea are set out in Table II, again ignoring the small amount of untransported ammonia.

Table II.

Actual urea mg.	Optimum p_H range of buffer	Average % of urea hydrolysed	Actual urea mg.	Optimum p_H range of buffer	Average % of urea hydrolysed
0·5	6·6–7·8	96·3	2·0	6·2–7·8	98·5
1·0	6·6–7·8	97·8	4·0	6·3–7·6	98·2
1·5	6·2–7·8	98·5	6·0	6·3–7·6	98·5

Summary.

1. A method of preparing a dry stable urease powder from jack beans is described.

2. The optimum initial p_H values for buffers in the urease hydrolysis of urea have been determined for amounts of urea varying from 0·5 to 6·0 mg.

3. For general purposes, including blood-urea estimations, a safe initial p_H range is 6·6–7·0 for quantities up to 6 mg. of urea.

4. This range is obtained by adding to KH_2PO_4 solution one-third to one-half of its equivalent of NaOH and adjusting the solution to contain the equivalent of 0·6 % of KH_2PO_4.

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CCXXXVIII. STUDIES OF THE COMPLEXES OF IRON WITH VARIOUS BIOLOGICAL MATERIALS.

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(*Received August 21st, 1934.*)

In a previous communication the present writer [1934, 1] has confirmed Hill's observation [1931] that all the iron of the hen's egg is in the inorganic form. It was found that when the proteins of a suspension of egg or egg-yolk were precipitated with trichloroacetic acid, no iron was detected in the filtrate. Iron was also absent from the filtrate after the addition of inorganic iron to the egg in the form of iron alum. When, however, thiolacetic acid was added before the precipitation of the proteins, iron was found in the filtrates.

The iron content of the filtrates was determined colorimetrically by means of the colour produced by iron and thiolacetic acid in alkaline solution [Lyons, 1927; Tompsett, 1934, 1]. The iron content of the egg as determined on the filtrates by this method was found to give the same value within the limits of experimental error as that obtained after ashing. It appears that iron forms very stable complexes with the proteins or other organic constituents of the hen's egg. Similar phenomena were noticed in the determination of the inorganic iron content of whole blood and serum. Copper did not appear to form such complexes. The writer has already shown [1934, 2] that, when the proteins of whole blood or serum are precipitated with trichloroacetic acid, the whole of the copper is present in the filtrate and added copper may be determined quantitatively.

Lintzel [1931] and Heubner [1926] have stated that ferric but not ferrous iron forms stable complexes with proteins and therefore suggest that iron must be reduced to the ferrous condition before absorption from the intestine. As thiolacetic acid liberates iron from its stable complexes and reduces ferric iron to the ferrous condition, in all probability complexes are not formed with ferrous iron.

This communication is an extension of earlier work instituted to determine the possibility of substances other than thiolacetic acid liberating iron from its complexes and to attempt to study the nature of the substances which apparently form the complexes. Sodium hydrosulphite was used owing to the ease with which it reduces ferric iron to the ferrous condition; also sodium pyrophosphate since it forms un-ionised complexes with iron and is an important constituent of muscle.

Experimental.

All filter-papers used in this investigation were washed with dilute acid and distilled water and dried, and the trichloroacetic acid was distilled *in vacuo*. The sodium pyrophosphate was found to be free from iron in the amounts used. The sodium hydrosulphite contained an appreciable amount of iron which was allowed for.

Egg-yolk was first studied. The yolk of one egg was diluted to 100 ml. Four 10 ml. samples were taken and treated as follows:

(1) Control diluted to 15 ml. with distilled water.

(2) 2 ml. thiolacetic acid added and distilled water to 15 ml.

(3) 5 ml. of 4 % sodium pyrophosphate added and distilled water to 15 ml.

(4) 2·5 ml. of 5 % sodium hydrosulphite added and distilled water to 15 ml.

After standing 5 minutes, 15 ml. of 20 % trichloroacetic acid were added, well shaken and filtered. To 5 ml. of filtrate 6 drops of thiolacetic acid were added followed by 1 ml. ammonia, sp. gr. 0·880. The colours were compared in a colorimeter with standards containing 0·015 mg. Fe similarly prepared. In the case of the controls negative reactions for iron were obtained. A precipitate of calcium phosphate appeared in the determinations containing pyrophosphate but this was separated by centrifuging without removing any of the colour. When sodium hydrosulphite was used the colour did not appear at once in either the test or the standard but only after vigorous shaking and standing. A precipitate of calcium phosphate appeared when sodium hydrosulphite was used to liberate the iron but could be removed by centrifuging without interfering with the estimation. When thiolacetic acid was used to liberate the iron no precipitate was ever obtained.

The results of these experiments are shown in Table I. It will be seen that within the limits of experimental error the iron content of egg-yolk is found to be the same whether thiolacetic acid, sodium pyrophosphate or sodium hydrosulphite is used to liberate the iron from its complex. Experiments were also carried out using sodium glycerophosphate and dithioglycollic acid but the filtrates gave negative tests for iron.

Table I. *The iron content of egg-yolk (expressed as mg. Fe per egg).*

	Thiolacetic acid	Sodium pyrophosphate	Sodium hydrosulphite	Control
1	1·28	1·24	1·22	Nil
2	1·24	1·18	1·26	Nil
3	0·96	0·99	1·00	Nil
4	1·10	1·12	1·17	Nil
5	1·16	1·12	1·10	Nil

In the second series of experiments ferric iron was added to various biological materials. Milk, sodium caseinogenate and egg-white gave negative reactions for iron within the limits of the amount used. Lecithin, gelatin and edestin contained appreciable amounts which were allowed for. The lecithin emulsion was prepared by pouring an ethereal solution of lecithin into boiling water and boiling off traces of ether. The sodium caseinogenate was made up in 2 % solution by dissolving a commercial preparation in boiling water and then cooling. The egg-white solution was prepared by diluting the white of a hen's egg to 100 ml. The edestin solution was prepared in a concentration of 2 % in 0·1 *N* HCl. The gelatin solution was prepared in a concentration of 2 % in hot water and cooled. Ferric iron in the form of iron alum was added to these solutions and 10 ml. samples treated in the same way as the egg-yolk samples described in the previous experiments. The results are shown in Table II and are expressed in mg. Fe per 10 ml. solution or suspension. In the experiments with milk a precipitate of calcium phosphate appeared which could be removed by centrifuging without interfering with the depth of colour.

Table II. *Recovery of iron added to various biological materials.*

		Ferric iron. Iron recovered (mg.)			
	Iron added mg.	Control	Thiolacetic acid	Sodium pyro-phosphate	Sodium hydro-sulphite
1 % lecithin suspension	0·10	Nil	0·099	0·097	0·096
	0·05	Nil	0·052	0·054	0·051
2 % sodium caseinogenate	0·10	Nil	0·096	0·102	0·105
	0·05	Nil	0·052	0·053	0·051
Milk	0·10	Nil	0·106	0·105	0·107
	0·05	Nil	0·053	0·047	0·048
Egg-white	0·10	0·091	—	—	—
	0·10	0·081	0·102	0·106	0·098
	0·15	0·124	—	—	—
	0·10	0·078	0·106	0·107	0·103
2 % edestin	0·10	0·099	—	—	—
	0·15	0·145	—	—	—
	0·20	0·193	—	—	—
2 % gelatin	0·10	0·094	—	—	—
	0·15	0·144	—	—	—
	0·20	0·198	—	—	—

It will be seen that whereas no iron is present in the control filtrates of milk, lecithin or sodium caseinogenate, it is present in the filtrates after treatment of these materials with thiolacetic acid, sodium hydrosulphite or pyrophosphate, the iron being liberated quantitatively by these reagents. It appears that ferric iron forms complexes with these substances, similar to those formed with egg-yolk. In the case of egg-white however iron did appear in the control filtrates and in some cases almost quantitatively. Mixtures of egg-white and ferric iron were tested directly with $\alpha\alpha'$-dipyridyl [*cf.* Hill, 1931] and were found to contain ferric iron, no signs of any reduction to ferrous iron being noted. In the cases of edestin and gelatin iron appeared in the control filtrates quantitatively. Gelatin did not appear to be completely precipitated with the concentration of trichloroacetic acid used in these experiments. It has been mentioned previously that the gelatin, edestin and lecithin contained appreciable amounts of iron. In the cases of edestin and gelatin this iron appeared quantitatively in the filtrates after precipitation of the proteins with trichloroacetic acid. With lecithin however no iron appeared in the filtrate until the phosphatide had been treated with thiolacetic acid, sodium hydrosulphite or pyrophosphate before precipitation with trichloroacetic acid.

Experiments were carried out to test the recovery of ferrous iron added to these biological materials. Using $\alpha\alpha'$-dipyridyl it was found that under the conditions of the experiments ferrous iron was oxidised very quickly to the ferric condition in the presence of these substances. Under these conditions results identical with those obtained in Table II were obtained.

From the data which have been obtained it appears that ferric iron forms complexes with many biological substances. Since thiolacetic acid and sodium hydrosulphite reduce ferric iron to the ferrous state and also liberate ferric iron from its complexes, it seems that ferrous iron does not form such complexes. Although ferric iron forms complexes with sodium caseinogenate, lecithin, milk and egg-yolk it does not appear to form these with egg-white, edestin or gelatin. The slight loss of iron in the filtrates when the proteins of egg-white are precipitated with trichloroacetic acid is probably due to traces of lecithin which

egg-white invariably contains. This suggests that ferric iron forms complexes not with simple proteins but with non-diffusible phosphorus compounds, *e.g.* phosphatides and phosphoproteins. Of the non-reducing substances examined, pyrophosphate was the only one liberating ferric iron from its complexes. This is probably due to its property of forming un-ionised ferric pyrophosphate. The question naturally arises as to whether pyrophosphates play any part in the absorption and transport of iron in the human body.

The recovery of added copper.

Since iron and copper are closely related, the latter apparently influencing the formation of haemoglobin from the former, the recovery of copper added to milk, 2 % sodium caseinogenate, egg-yolk, egg-white and 1 % lecithin suspension was examined. Copper was added in the form of copper sulphate to these solutions or suspensions and the copper determined directly in the trichloroacetic acid filtrates by the method described by the writer [1934, 1] for blood. In the cases of egg-yolk and egg-white allowances were made for their initial copper contents but in the cases of the other substances negative reactions for copper were obtained in the amounts used. The results are shown in Table III where it will be seen that added copper could be determined quantitatively in the filtrates.

Table III. *Recovery of copper added to various biological materials.*

	Copper added mg.	Copper recovered mg.
Egg-yolk (1)	1·125	1·118
(2)	0·125	0·117
Egg-white (1)	0·100	0·095
(2)	0·100	0·093
10 ml. milk (1)	0·025	0·024
(2)	0·025	0·024
(3)	0·050	0·048
10 ml. 2 % sodium caseinogenate (1)	0·025	0·026
(2)	0·025	0·026
(3)	0·050	0·051
10 ml. 1 % lecithin suspension (1)	0·025	0·027
(2)	0·025	0·023
(3)	0·050	0·051

The initial copper contents of the two whole eggs were 0·06 and 0·08 mg. per egg respectively. These results are of the same order as obtained by Loeschke [1931] who obtained values of 0·055–0·095 mg. per egg, and McFarlane *et al.* [1930] who obtained an average value of 0·08 mg. per egg. The yolks contained slightly more copper than the whites.

The inorganic iron of serum and whole blood.

A method has been described for the determination of the inorganic iron content of whole blood [Tompsett, 1934, 1]. It has been found that, with blood also, pyrophosphate can be used in place of thiolacetic acid to liberate the inorganic iron from its complexes.

The condition of the inorganic iron of the plasma or corpuscles does not appear to have been studied. Any method involving several manipulations is unsuitable owing to the ease with which ferrous iron is oxidised to the ferric state. Any test used must be applied directly to the material. The most suitable

reagent appears to be $\alpha\alpha'$-dipyridyl which gives a red colour with ferrous and no colour with ferric iron. It gives no reaction with haematin-like substances. The test was applied directly to serum in the manner used by Hill [1931] for egg-yolk but no colour appeared. On the addition of a reducing substance, *e.g.* sodium hydrosulphite or thiolacetic acid, a red colour appeared. It appears that the inorganic iron of serum is in the ferric state but if on the other hand it is in the ferrous state it is extremely doubtful whether it would remain in this condition long after the removal of the blood from the body. In the case of whole blood, after the addition of $\alpha\alpha'$-dipyridyl sufficient alcohol was added to precipitate the proteins completely. A control was carried out at the same time but without the addition of $\alpha\alpha'$-dipyridyl. The filtrate showed a decided reddish colour when compared with the control filtrate. This was obtained in every case examined and suggests that part at least of the inorganic iron of the corpuscles is in the ferrous state.

SUMMARY.

1. Ferric iron has been shown to form complexes with certain biological materials. It is liberated from these complexes by reducing substances such as thiolacetic acid and sodium hydrosulphite. Ferrous iron does not appear to form such complexes.

2. Ferric iron is also liberated from these complexes by sodium pyrophosphate. This does not involve a reduction but is more probably due to the formation of un-ionised ferric pyrophosphate.

3. Ferric iron appears to form complexes with the non-diffusible phosphorus compounds, *e.g.* phosphatides and phosphoproteins, and not with the simple proteins, *e.g.* egg-white, edestin and gelatin.

4. Copper does not form complexes with any of these substances.

5. Part at least of the inorganic iron of the corpuscles of blood is in the ferrous condition.

In conclusion I wish to thank Dr D. P. Cuthbertson for his advice and criticism and the Medical Research Council for a grant towards expenses.

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CCXXXIX. THE FATTY ACIDS OF PIG LIVER. II. FURTHER EVIDENCE ON THE OCTADECENOIC ACIDS AND THE DESATURATION THEORY.

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(*Received August 31st, 1934.*)

In a recent paper in which we reported the results of investigations into the chemical nature of the octadecenoic acids of pig liver the conclusion was reached that at least 85 % of these were present as the $\Delta^{9:10}$ compound, while the remaining 15 % did not contain any $\Delta^{12:13}$ acid [Channon *et al.*, 1934]. This result was contrary to that obtained in a similar study by Hartley [1909], for the only octadecenoic acid found by him was the $\Delta^{12:13}$ derivative. The disagreement in these results is of importance, for that of Hartley has provided one of the main lines of evidence on which the theory of the desaturation of fatty acids in the liver is based.

Bearing in mind the large quantities of pig liver used in the preparation of the fatty acids both by Hartley and by ourselves and taking into consideration the number of animals concerned, it seems at least improbable that the divergence in our respective results can be attributed to a fundamental difference in the composition of the original acids. It is true that neither Hartley nor ourselves paid attention to the previous diet of the animals used, but present knowledge of the appearance of the fatty acids of the food in the liver does not suggest that any particular acid would appear in the liver to the complete exclusion of others of similar chemical nature, even if that acid were present in the food in considerable amount. Further, as far as we are aware, the presence of $\Delta^{12:13}$ octadecenoic acid has not been reported in natural products except by Hartley. Hence disregarding the possibility of chemical idiosyncrasy in the respective groups of animals, these considerations have led us to attempt to find an explanation of these contradictory results on the basis that our somewhat different methods of preparation of the hydroxy-acids and of their further oxidation might have caused different products to be formed. We are aware, of course, that Hartley had no difficulty in identifying the $\Delta^{9:10}$ compound as the only octadecenoic acid in lard, and that by similar methods he failed to find this acid in liver. Nevertheless it seemed important to investigate the matter further by carrying out the various stages exactly as Hartley had done in order that the results might be compared, and we were fortunate in having at our disposal sufficient of the original acids used by us in our previous investigations to make this possible.

We have accordingly investigated both the possibilities that the disagreement in the results might be due to some change taking place either in the further oxidation of the dihydroxystearic acid or in the preparation of that

product from the original acids. To test the former of these 5 g. of the higher-melting dihydroxystearic acid, prepared from the liver acids and proved by a recognised method to be a 9 : 10 derivative as described in our original paper, were oxidised exactly as described by Hartley, who used the method of Edmed [1898]. We had no difficulty in isolating *n*-octanoic and suberic acids, as the main products of oxidation. Further, when 112 g. of the original liver acids were treated throughout as described by Hartley, we were again able to isolate these same two products.

Hence our original finding that the main octadecenoic acid of pig liver is the $\Delta^{9:10}$ compound was confirmed by carrying out the oxidations according to Hartley's procedure. It has to be borne in mind however that Hartley's recognition of the presence of the $\Delta^{12:13}$ acid was based on partial evidence only. He prepared and then oxidised the dihydroxystearic acid and removed the volatile acid products by distillation in steam. Calcium and barium salts were prepared from the steam distillate, and these salts were found to contain the percentage of calcium and barium required for the corresponding salts of hexanoic acid. This monobasic acid was not further characterised. Nor was the dibasic acid, which must necessarily have been formed at the same time, isolated or identified in any way. Hartley suggested that his dibasic acid had either (*a*) been oxidised to oxalic acid which he found in considerable quantity as a product of oxidation, or (*b*) that it was so similar in properties to the original unchanged dihydroxystearic acid as to be inseparable from it in the amounts which he had available. From our experiments carried out at a temperature of 80° quite good yields of the crude dibasic acid were obtained, and the occurrence of oxalic acid has been explained by Lapworth and Mottram [1925, 2] who investigated the work of Edmed more fully. Edmed stated that under his conditions (identical with those used by Hartley), $CH_3.(CH_2)_7.CHOH.CHOH.(CH_2)_7.COOH$ (a 9 : 10 derivative) yielded $CH_3.(CH_2)_7.COOH$ (nonanoic acid) and $HOOC.(CH_2)_7.COOH$ (azelaic acid). But when more fully investigated it was shown by Lapworth and Mottram that, although this type of oxidation does take place to some extent, another set of products is simultaneously formed by the cleavage taking place in such a way as to give $CH_3.(CH_2)_6.COOH$ (octanoic acid), $C_2H_2O_4$ (oxalic acid) and $HOOC.(CH_2)_6.COOH$ (suberic acid). Lapworth and Mottram did not indicate what proportion of the dihydroxystearic acid yields octanoic, suberic and oxalic acids when Edmed's method is used, but our results, in which we obtained octanoic and suberic acids as the main products by both methods, indicated that the main oxidation is still of this latter type. If that be true in all cases, irrespective of the position of the double bond, then the finding of hexanoic acid as a main product of oxidation simultaneously with oxalic acid by Hartley would not indicate the presence of a $\Delta^{12:13}$ acid, but would suggest the presence of a $\Delta^{11:12}$ compound, thus: $CH_3.(CH_2)_5.CH(OH).CH(OH).(CH_2)_9COOH$ (*i.e.* 11 : 12) would yield as the main products $CH_3.(CH_2)_4.COOH$ (hexanoic acid), $C_2H_2O_4$ (oxalic acid) and $HOOC.(CH_2)_8.COOH$ (sebacic acid). In this connection it is interesting to observe that from the 15 % or so of lower-melting dihydroxystearic acid prepared by fractional crystallisation of the main bulk as described in our previous communication, we obtained a certain small amount of sebacic acid; the monobasic acid however, at all times difficult to characterise definitely when present, as in this case, in a mixture, appeared to be heptanoic rather than hexanoic acid. This fact, taken in conjunction with the first part of our present discussion, does not suggest that any explanation can be given along these lines for the finding by Hartley of hexanoic acid, particularly when it is realised that he was able to show by identical methods

and without difficulty that the octadecenoic acid of lard was the usual $\Delta^{9:10}$ compound.

Unfortunately further information cannot be obtained from the melting-point of the dihydroxy-acid itself for the effect of the position of the two hydroxy-groups upon the melting-point is often too slight to make this factor a reliable indication unless an adequate amount of material is available to enable rigorous purification to be accomplished.

We ourselves have had two very great advantages in carrying out this work. In the first place, we have been able to make use of the method initially suggested by Robinson and Robinson [1925] and elaborated by Lapworth and Mottram [1925, 1] for the preparation of the hydroxy-acids—a method which gave from seven to eight times the yield obtainable by the procedure available to Hartley in 1909. In the second place, we have had very much larger quantities of material on which to work. Perhaps these two facts alone might be sufficient to account for the divergence in the two sets of results.

Our experiments described below therefore throw no further light on the problem under discussion, except to confirm our previous work and to show that the disagreement between the results of Hartley and ourselves is not due to the different oxidation technique employed.

Experimental.

I. *Oxidation of dihydroxystearic acid prepared by the method of Lapworth and Mottram [1925, 1] and oxidised by the method of Hartley [1909].*

5 g. dihydroxy-derivative, m.p. 130·5–131°, obtained from pig liver as described in our previous communication, were dissolved in 166 ml. water containing 3·31 g. potassium hydroxide. The solution was heated to 80°, and 400 ml. water containing 5·3 g. potassium permanganate were added, the mixture being shaken and then allowed to cool. After removal of the manganese dioxide by filtration, the colourless filtrate was acidified with 10 % H_2SO_4, when a voluminous white precipitate was thrown down. This weighed 3·8 g. and consisted mainly of unchanged dihydroxystearic acid. This material was reoxidised as before, and again all the permanganate was decolorised and some unchanged substance recovered. The latter was oxidised under the same conditions a third time, when no dihydroxystearic acid remained unchanged and the permanganate was definitely in excess.

The monobasic acid. As was done by Hartley, the filtrate from the unchanged dihydroxy-derivative after the first oxidation was distilled in steam. The distillate was exactly neutralised (36 ml. 0·1 *N* NaOH being required), and evaporated to a small bulk. On adding calcium chloride solution, a white precipitate was formed. This calcium salt was filtered off, washed and dried. It weighed 0·48 g. (Found by ignition: Ca, 12·30 %. $(C_6H_{11}O_2)_2$ Ca requires Ca, 14·8 %; $(C_8H_{15}O_2)_2$ Ca requires Ca, 12·27 %. Hartley [1909] found: Ca, 14·7 %.) As confirmation the calcium oxide from a second analysis of 0·1734 g. was washed into a small flask with hydrochloric acid and precipitated as oxalate, the latter being titrated with permanganate. 10·35 ml. *N*/10 permanganate solution were required. Whence Ca = 11·9 %.

It is therefore clear that the lowest monobasic acid formed by either method is in our case octanoic acid and that the hexanoic acid found by Hartley on similar evidence is absent. This conclusion is confirmed by studying the dibasic acid.

The dibasic acid. The residue left after distilling all the three oxidation-liquors in steam was concentrated to about 50 ml. when 1·28 g. dibasic acid,

M.P. 135°, crystallised out. A second crop was obtained, weighing 0·15 g., M.P. 102–118°. This probably consisted of crude azelaic and suberic acids mixed. The first crop was recrystallised, yielding 0·63 g. (C, 55·1; H, 8·0 %; M.P. 141·5°; mol. wt. 174·6. Suberic acid, $C_8H_{14}O_4$, requires C, 55·15; H, 8·05 %; M.P. 141°; mol. wt. 174.) A small third crop of very crude dibasic acid (0·2 g.) was also obtained. The total yield corresponds approximately to 60 % of the theory for suberic acid. The isolation of this particular dibasic acid alone suggests that the method adopted for the oxidation plays no part in accounting for the difference in the results obtained by Hartley and by ourselves.

II (*a*). *The preparation of dihydroxystearic acid by Hartley's method.*

By this procedure the Twitchell process used by us in the original work was not carried out. 112 g. of the original mixed liver acids (I.V. 110, mol. wt. 300) were mixed with 112 ml. potassium hydroxide solution (sp. gr. 1·145), and the mixture was dissolved in 4·6 l. of water cooled to 0°. 4·6 l. 1·5 % potassium permanganate also at 0° were added slowly with vigorous shaking. After 10 minutes a saturated solution of sodium bisulphite was added and the whole acidified with hydrochloric acid. The white precipitate so formed was filtered off, dried in a vacuum-desiccator and extracted as usual with light petroleum. The latter extracted 35·0 g. acids (I.V. 36). This corresponds well with Hartley's results at this stage.

The portion insoluble in light petroleum was washed free from a brown gummy substance by cold ether. From the residue consisting of di- and tetrahydroxystearic acids, the dihydroxy-acid was removed by extraction with warm ether. The total yield of the crude dihydroxy-derivative amounted to 2·2 g., M.P. 125° (indef.). After one recrystallisation from 70 % alcohol (charcoal) the M.P. rose to 127°. This yield of approximately 2 % corresponds to that of 15 % obtained by the more recent methods previously used by us on the same batch of liver acids.

Lest any of this product should be lost in further processes of purification it was oxidised immediately without further treatment.

II (*b*). *Oxidation of the dihydroxy-derivative so formed by Hartley's method.*

1·5 g. of this substance were oxidised according to the details given in section I above, except that only one oxidation was carried out [*cf.* Hartley, 1909, p. 364]. Only a portion of the 1·5 g. was therefore affected, which accounts in part for the lower yields of oxidation products. This enabled the use of bisulphite to be avoided, because all the permanganate was used up, and there was thus no chance of sulphite interfering at the end in the formation and analysis of the calcium salt.

The monobasic acid. The manganese dioxide and then the unchanged dihydroxystearic acid were filtered off, the filtrate distilled in steam, the distillate neutralised exactly and evaporated to a small bulk. After adding calcium chloride, the calcium salt was filtered off. It weighed 0·15 g. On ignition 0·1463 g. gave 0·0248 g. CaO. Whence Ca = 12·1 %. (Theory 12·27 %.) In confirmation the Ca in this CaO was estimated by the oxalate-permanganate method. 8·80 ml. *N*/10 permanganate were required; whence Ca = 12·0 %.

The dibasic acid. This was obtained in two crops: (1) 0·23 g., M.P. 127° (indef.) and (2) 0·14 g., M.P. 122–128°. These were combined and recrystallised, and some oily product removed.

First crop 0·07 g., M.P. 139°. (Found: C, 54·94; H, 8·06 %. Suberic acid ($C_8H_{14}O_4$) requires C, 55·15; H, 8·05 %.)

It is therefore again quite certain that the products of oxidation contain octanoic and suberic acids and that the different methods adopted are not the source of the disagreement in results.

SUMMARY.

In a previous paper [1934] it was shown that at least 85 % of the octadecenoic acids of pig liver is the $\Delta^{9:10}$ compound. This result is at variance with that of Hartley [1909] who found the $\Delta^{12:13}$ acid as the sole compound of this nature present. In an effort to explain these conflicting results a further sample of the original liver acids has been submitted to treatment identical with that used by Hartley. The monobasic and dibasic acids isolated as further oxidation products of the dihydroxy-derivatives were octanoic and suberic acids, confirming our original finding that the main octadecenoic acid is the $\Delta^{9:10}$ compound.

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CCXL. STUDIES ON REVERSIBLE DEHYDROGENASE SYSTEMS.

III. CARRIER-LINKED REACTIONS BETWEEN ISOLATED DEHYDROGENASES.

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(*Received September 1st, 1934.*)

Whilst the study of the aerobic oxidation of organic substrates by cells has been prosecuted with increasing intensity during the last two decades, the mechanism of anaerobic oxidations has received scanty attention. In a series of papers by Quastel and Stephenson [1925], Quastel, Stephenson and Whetham [1925] and Quastel and Wooldridge [1929] the experimental groundwork was laid for a clear conception of the systems involved in anaerobic oxidations. The important facts and conclusions of these authors are the following. (1) Substances may be divided into two classes, those that act as hydrogen donators and those that act as hydrogen acceptors. (2) Reactions between certain hydrogen donators and acceptors are catalysed by bacteria. For example, in the presence of resting *Bact. coli* lactate and nitrate can react to form pyruvate and nitrite, and lactate and fumarate can react to form pyruvate and succinate. In some cases the same substance could function as either donator or acceptor of hydrogen, according to the conditions of the experiment. (3) Bacteria can grow anaerobically on a medium containing only a hydrogen donator and a hydrogen acceptor, provided that one of the substances can enter into the synthetic reactions of the cell. (4) The reaction between donator and acceptor is catalysed by specific dehydrogenase systems and does not proceed in the absence of the enzymes. (5) The suggestion was made that the reaction between dehydrogenase systems is not direct but proceeds through some intermediary in the cell, presumably a substance of the type of an oxidation-reduction indicator.

These pioneer investigations of Quastel and his collaborators described the oxidative reactions which take place anaerobically in bacteria but left unanswered the question of mechanism. We may conceive of two enzymes in such close proximity on the cell surface that the reaction between the two substrates is a direct one, or we may picture a random distribution of enzymes in the cell with diffusible carriers linking the reducing and oxidising systems. In this paper evidence will be presented that isolated dehydrogenase systems do not react directly but require the intermediation of some carrier which can be reduced by the negative system and oxidised by the positive system. The question of what substances in either animal cells or bacteria can function as carriers will also be considered.

Schott and Borsook [1933] in a brief note indicated that toluene-treated *Bact. coli* could not carry out the reaction between either formate and pyruvate

or lactate and fumarate, but that on addition of an oxidation-reduction indicator these reactions did proceed. They applied the term "coupled reaction" to these anaerobic oxidations requiring a carrier, but the choice is unfortunate as the term has been previously used with a different meaning (*e.g.* the oxidation of nitrite to nitrate by peroxidase during the oxidation of hypoxanthine by xanthine oxidase and oxygen). To avoid any confusion of terminology, we propose the expression "carrier-linked reaction" to describe reactions between dehydrogenases which require an intermediary hydrogen carrier.

Experimental methods.

The following dehydrogenase preparations were used in the various experiments:

(1) *Xanthine oxidase.* The dried ammonium sulphate precipitate from whey was used direct [Dixon and Kodama, 1926].

(2) *Succinoxidase.* Succinoxidase was prepared from heart muscle according to the directions of Borsook and Schott [1931].

(3) *Glucose dehydrogenase.* The enzyme and co-enzyme were prepared from liver by the method of Harrison [1933].

(4) *Hexosemonophosphate dehydrogenase system.* The enzyme was prepared from bottom yeast and the co-enzyme from horse red blood cells as described by Warburg and Christian [1932]. The co-enzyme solution was used in the crude state.

(5) *Formic dehydrogenase and nitratase preparation.* This was made from washed *Bact. coli* by the method of Stickland [1929]. The preparation contains both formic dehydrogenase and nitratase (the enzyme which activates nitrate as a hydrogen acceptor).

It is noteworthy that the nitratase has not been detected previously in this preparation. This is the first demonstration of a cell-free nitratase preparation.

(6) *Lactic dehydrogenase and nitratase preparation.* Lactic dehydrogenase was prepared by the method of Stephenson [1928]. This also contains nitratase in addition.

(7) In a few experiments crude acetone-dried liver was used as a source of various enzymes (xanthine oxidase, succinic dehydrogenase and lactic dehydrogenase).

Preparations (1), (3) and (7) were kept as dry powders in a desiccator, and (2), (4), (5) and (6) in solution at 0–2°.

Methods of estimation.

All solutions were deproteinised by mixing with an equal volume of 1·5 % uranium acetate solution, and indicators were removed by adsorption on kieselguhr. Uric acid was determined colorimetrically with Benedict's reagent, and nitrite colorimetrically with the Griess-Ilosvay reagent. Pyruvic acid was estimated roughly by the nitroprusside test.

Experimental results.

Reactions between isolated dehydrogenase systems.

In the case of each pair of dehydrogenases to be tested, two Thunberg tubes were set up with identical solutions, except that to one was added 1 ml. of some oxidation-reduction indicator (at a concentration of the order of $M/2000$) and

to the other 1 ml. of water. Each tube contained in addition 1 ml. of $M/5$ phosphate buffer, 1 ml. of each enzyme preparation and 1 ml. of each substrate at a concentration of $M/10$ (except in the case of hypoxanthine, which was used at $0{\cdot}03\,M$). The tubes were evacuated thoroughly and incubated at 40° for several hours. At the end of the incubation period the solutions were deproteinised and freed from indicator, and the concentration of one of the products of the reaction was estimated. It was observed in all cases that no pair of dehydrogenases will react together except in the presence of an oxidation-reduction indicator. The results are given in Table I. With impure preparations

Table I.

Negative system	Indicator	Positive system	p_H	Time hours	Product estimated	Conc. of product M
Formic dehydrogenase + formate	Ethyl Capri blue	Nitratase + nitrate	7·0	3	Nitrite	1×10^{-3}
Lactic dehydrogenase + lactate	Ethyl Capri blue	Nitratase + nitrate	7·0	3	Nitrite	5×10^{-5}
Xanthine oxidase + hypoxanthine	Methyl viologen	Succinoxidase + fumarate	7·0	$3\frac{1}{2}$	Uric acid	$7{\cdot}5 \times 10^{-3}$
Succinoxidase + succinate	Methylene blue	Nitratase + nitrate	7·5	$5\frac{1}{2}$	Nitrite	1×10^{-4}
Lactic dehydrogenase + lactate	Ethyl Capri blue	Succinoxidase + fumarate	7·5	5	Pyruvate	2×10^{-3}
Xanthine oxidase + hypoxanthine	Benzyl viologen	Lactic dehydrogenase + pyruvate	7·0	5	Uric acid	5×10^{-3}
Hexosemonophosphate system	Pyocyanine	Nitratase + nitrate	7·0	4	Nitrite	1×10^{-4}
Glucose dehydrogenase + glucose	Ethyl Capri blue	Nitratase + nitrate	7·0	5	Nitrite	5×10^{-5}

a very slight reaction did occur in the absence of an added indicator, but with purified dehydrogenase solutions not the slightest direct reaction could be detected. Presumably impure preparations contain a minute amount of a natural carrier, though quite insufficient to allow the reaction to proceed at maximum velocity. But even in the case of the largest blank reaction that we have observed, the acceleration in the presence of a suitable indicator was at least 2000 %.

The function of the indicator.

Proof that the indicator functions by being reduced by the negative system and oxidised by the positive system is given by the following experiments. Thunberg tubes with hollow stoppers were used. The reducing system (1 ml. of enzyme preparation and 1 ml. of substrate), 1 ml. of buffer and 1 ml. of the indicator solution were placed in the main tube and the oxidising system (0·5 ml. of enzyme and 0·5 ml. of substrate) in the stopper. After thorough evacuation the tubes were incubated at 40° until maximum reduction of the dye was attained. Then the contents of the stopper were tipped in. In every case the indicator was either partly or completely reoxidised, the extent of reoxidation being obviously determined by the relative activities of the oxidising and reducing systems. Controls with enzyme alone in the stopper showed no trace of reoxidation of the indicator. The results are summarised in Table II.

Table II.

Negative system			Positive system	
Enzyme	Substrate	Indicator	Enzyme	Substrate
Formic dehydrogenase (*Bact. coli*)	Formate	Benzyl viologen	Nitratase (*Bact. coli*)	Nitrate
Lactic dehydrogenase (*Bact. coli*)	Lactate	Ethyl Capri blue	Nitratase (*Bact. coli*)	Nitrate
Xanthine oxidase (milk)	Hypoxanthine	Methyl viologen	Succinoxidase (heart muscle)	Fumarate
Hexosemonophosphate dehydrogenase (yeast)	Hexosemono-phosphate	Ethyl Capri blue	Succinoxidase (heart muscle)	Fumarate
Glucose dehydrogenase (liver)	Glucose	Ethyl Capri blue	Succinoxidase (heart muscle)	Fumarate
Xanthine oxidase (liver)	Hypoxanthine	Ethyl Capri blue	Succinoxidase (liver)	Fumarate
Lactic dehydrogenase (liver)	Lactate	Ethyl Capri blue	Succinoxidase (liver)	Fumarate
Xanthine oxidase (milk)	Hypoxanthine	Methyl viologen	Lactic dehydrogenase (*Bact. coli*)	Pyruvate

The kinetics of carrier-linked reactions.

Several aspects of the kinetics of these reactions have been studied in the cases of three pairs of dehydrogenase systems, *viz.* formate-nitrate, hypoxanthine-fumarate and lactate-nitrate.

(*a*) *The potential of the carrier.* It is evident that an indicator which is either more positive than the oxidising system or more negative than the reducing

Table III.

Indicator	E_0' at 30° and p_H 7 (v.)	Indicator	E_0' at 30° and p_H 7 (v.)
Phenolindophenol	+0·227	Nile blue	−0·116
Lauth's violet	+0·062	Janus green	−0·256
Cresyl blue	+0·047	Benzyl viologen	−0·359
Methylene blue	+0·011	Methyl viologen	−0·446
Ethyl Capri blue	−0·072		

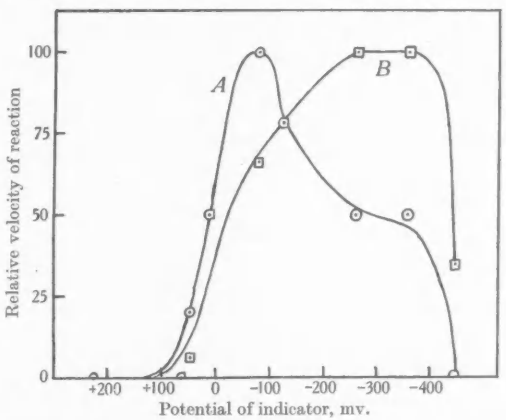

Fig. 1. Relation between potentials of indicators and their efficiency as carriers. System formate-nitrate, at p_H 7. Curves *A* and *B* represent different enzyme preparations.

system cannot function as a carrier. Theoretically any reversible indicator whose characteristic potential lies in the range between the two enzyme systems should be able to catalyse the interaction. Experiments were carried out to determine the relation between the potentials of indicators and their efficiency as carriers.

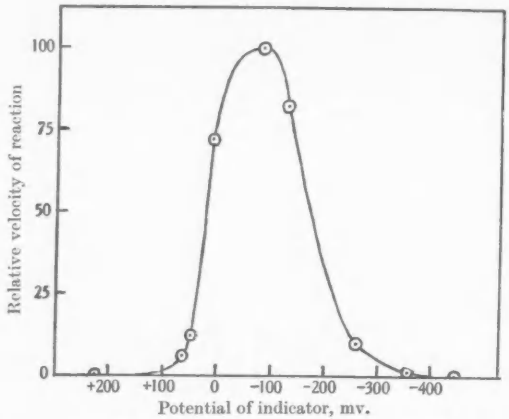

Fig. 2. Relation between potentials of indicators and their efficiency as carriers. System lactate-nitrate, at p_H 7.

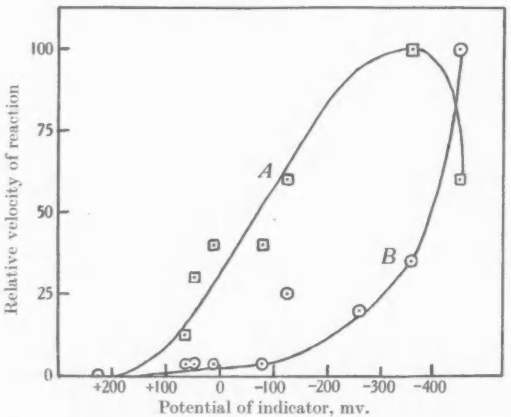

Fig. 3. Relation between potentials of indicators and their efficiency as carriers. System hypoxanthine-fumarate, at p_H 7. Curves *A* and *B* represent different preparations of succinoxidase.

The series of indicators used covered the range from r_H 0 to r_H 22 in convenient steps, as is shown in Table III. The experiments were carried out exactly as previously described, except that in a series of ten tubes one contained no indicator and the other nine the various indicators at a concentration of $M/10{,}000$. The concentration of one product of the reaction was estimated after from 3 to 5 hours' incubation at 40°. The results are shown in Figs. 1, 2 and 3.

It is clear that with any particular pair of dehydrogenase systems a rough relation exists between potential and the efficiency of a carrier. In every case a maximum velocity is observed at some point on the potential scale between the potentials of the two enzymes concerned, though sometimes the maximum

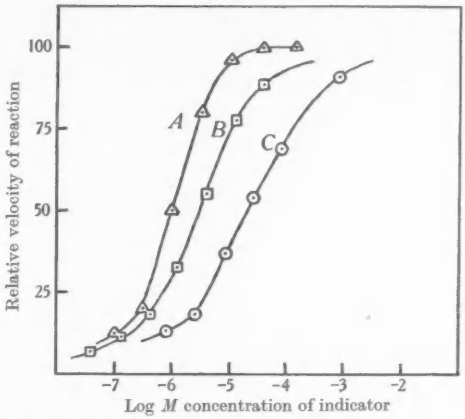

Fig. 4. Effect of dilution of indicators on the rate of reaction between formate and nitrate. Curve *A*, Nile blue; *B*, ethyl Capri blue; *C*, benzyl viologen.

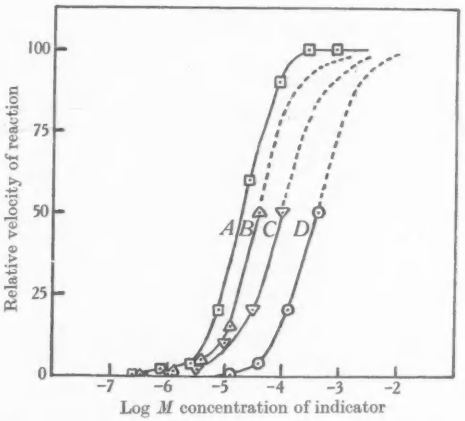

Fig. 5. Effect of dilution of indicators on the rate of reaction between hypoxanthine and fumarate. Curve *A*, benzyl viologen; *B*, ethyl Capri blue; *C*, methylene blue; *D*, methyl viologen.

covers a fairly wide range. There is always a decrease in velocity on each side of the maximum, until a zero value is reached with indicators which lie outside the range of potential of the two enzyme systems. The potential-velocity curves are naturally different for different pairs of enzymes and also differ with different preparations of the same enzymes. The significance and interpretation of these results will be discussed later.

(*b*) *The concentration of the carrier.* Experiments were carried out in the usual way, with a series of dilutions of the indicator. Figs. 4 and 5 show the effect of dilution of the carrier on the velocity of the reaction between dehydrogenases. It will be seen that as a rule the indicators can function as carriers at a very low concentration, especially in the case of the system formate-nitrate. With this system Nile blue and Capri blue show a definitely positive result at a concentration of $3 \times 10^{-7} M$, but the fumarate-hypoxanthine system requires in general higher concentrations. It is noteworthy that the dyes function even at dilutions at which their colours cannot be detected. Discussion of these results will be found later in the paper.

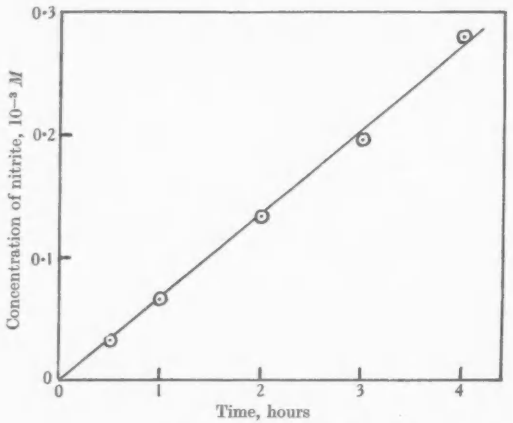

Fig. 6. Course of the reaction between formate and nitrate, with Nile blue as carrier.

(*c*) *The course of the reaction.* The reaction between formate and nitrate, linked by Nile blue, proceeds linearly. Five tubes were set up with the usual enzymes, substrates, buffer and indicator solutions, and the concentration of nitrite in the solutions was estimated after various time intervals. The result is shown in Fig. 6, and it justifies the use of single estimations as a measure of relative velocities.

(*d*) *Specificity.* It is noteworthy that all the members of the indicator series used to test the relation between the potential and the efficiency of a carrier were basic in character. If acid indicators were included, such as the indigosulphonates and rosindulin, they were found in every case not to fall on the same curve as the basic indicators (see Table IV). The general rule is that acidic indicators are reduced much more slowly than basic ones of the same potential. We may therefore conclude that a relation between potential and velocity will only hold for an indicator series all the members of which are basic, that is, chemical specificity as well as potential plays a part in determining the efficiency of a dye as a carrier. A striking example of specificity is that of pyocyanine, which in the cases of the formate-nitrate system (Table IV) and the hypoxanthine-fumarate system (Table V) is considerably more active as a carrier than its position on the potential scale should warrant.

Table IV. *Acidic indicators and pyocyanine tested as carriers, with the formate-nitrate system ($1\frac{1}{4}$ hours, 40°).*

Indicator	E_0' at 30° and p_H 7 (v.)	Nature of indicator	Relative speed of reaction
Methylene blue	+0·011	Basic	30
Pyocyanine	−0·034	Basic	130
Indigo tetrasulphonate	−0·046	Acidic	4
Capri blue	−0·076	Basic	90
Indigo trisulphonate	−0·081	Acidic	2
Nile blue	−0·125	Basic	100
Janus green	−0·258	Basic	80
Rosindulin	−0·282	Acidic	4
Neutral red	−0·325	Basic	4
Benzyl viologen	−0·359	Basic	80

The speed with Nile blue, the optimum of the normal series of indicators, is taken as the standard.

Table V.

Indicator	E_0' at 30° and p_H 7 (v.)	Relative speed of reaction
Methylene blue	+0·011	2
Pyocyanine	−0·034	140
Capri blue	−0·072	2
Nile blue	−0·125	25
Benzyl viologen	−0·359	40
Methyl viologen	−0·446	100

Reaction hypoxanthine-fumarate; $3\frac{1}{2}$ hours, 40°. The speed with methyl viologen, the optimum of the normal series of indicators, is taken as standard.

Natural hydrogen carriers.

Many substances derived from cells of animals, plants, moulds and bacteria have been described which are capable of reversible oxidation and reduction. The possibility is obvious that these substances might catalyse carrier-linked reactions in the same way as do artificial indicators. Tests were made with glutathione, ascorbic acid, cytochrome, pyocyanine, the yellow pigment of Warburg, lactoflavin, citrinin and ovoflavin, and the results are shown in Table VI. In the case of the Warburg hexosemonophosphate dehydrogenase system, experiments were carried out in Barcroft manometers to show that the

Table VI.

Enzyme systems: Positive	Enzyme systems: Negative	Carrier *Ca.* $M/1000$	Result
Nitratase-nitrate	Formic dehydrogenase-formate	Glutathione	−
		Ascorbic acid	−
		Cytochrome *c*	−
		Yellow pigment	−
		Lactoflavin	−
		Ovoflavin	−
		Citrinin	−
		Pyocyanine	+ + + +
Succinoxidase-succinate	Xanthine oxidase-hypoxanthine	Cytochrome *c*	−
		Lactoflavin	−
		Yellow pigment	−
		Pyocyanine	+ + + +
Nitratase-nitrate	Hexosemonophosphate-dehydrogenase system	Cytochrome *c*	−
		Lactoflavin	−
		Yellow pigment	(+)?
		Pyocyanine	+ + + +

various components of the system were active (see Table VII). The extraordinary conclusion follows from these results that none of the known natural oxidation-reduction indicators, pyocyanine excepted, can function as a carrier in linked reactions between the pairs of dehydrogenases studied.

Table VII.

	O_2 uptake in 3 hours (μl.)
Enzyme + yellow pigment + hexosemonophosphate	0
Enzyme + co-enzyme + hexosemonophosphate	0
Enzyme + co-enzyme + yellow pigment + hexosemonophosphate	120
Enzyme + co-enzyme + pyocyanine + hexosemonophosphate	35

Warburg and Christian [1933] have suggested that the yellow pigment plays no part in aerobic processes, but is directly concerned in anaerobic oxidations. The above results (Table VI) show clearly that there is no experimental basis for this suggestion.

Attempts to extract natural carriers from cells.

We have tried by mechanical, physical and chemical means to prepare from cells extracts which will function as carriers in linking dehydrogenases. Bacteria (*Bact. coli*, *Bact. dispar*, *M. lysodeikticus* and *S. lutea*), yeast and animal tissues (liver and heart muscle) have been employed, and the extracts prepared have been tested with the formate-nitrate and the hypoxanthine-fumarate systems. So far no active extracts have been obtained.

Velocity of carrier-linked reactions.

The velocity of these reactions will obviously depend on the activity of the less active of the pair of enzymes. The observed velocities are of the order of magnitude that would be expected from the activity of the weaker enzyme preparation, as the following examples show:

(1) 1 ml. succinoxidase + succinate reduced 1 ml. of $M/2000$ methylene blue in 6 minutes. $\therefore$ rate = 5 ml. of $M/1000$ per hour.

1 ml. of the same preparation, linked with excess of xanthine oxidase + hypoxanthine, produced 5 ml. of $M/130$ uric acid in 4 hours.

$\therefore$ rate = 10 ml. of $M/1000$ per hour.

(2) 1 ml. lactic dehydrogenase + lactate reduced 1 ml. of $M/5000$ ethyl Capri blue in 10 minutes.

$\therefore$ rate = 1 ml. of $M/1000$ per hour.

1 ml. of the same preparation linked with excess of succinoxidase + fumarate produced 5 ml. of $M/500$ pyruvate in 5 hours.

$\therefore$ rate = 2 ml. of $M/1000$ per hour.

Discussion.

General.

The object of the work to which the present paper is an introduction is to decide whether, in intact cells, dehydrogenases react together directly or through the intervention of diffusible hydrogen carriers. The evidence produced so far shows conclusively that dehydrogenases, when isolated from cell structure, in every case fail to react together, but can be made to do so by the addition of an appropriate artificial hydrogen carrier.

The only alternative to the theory that in normal cells the dehydrogenases are linked by means of natural diffusible hydrogen carriers is that the enzymes are placed so close to one another that the activated substrate molecules react directly (the possibility of the enzymes themselves being freely diffusible in the inside of the cells is ruled out by the fact that they fail to react directly when extracted from the cell). This is a difficult conception, which, to explain the complete non-specificity of reaction between all hydrogen donators and all hydrogen acceptors, involves one of the two assumptions: (*a*) that molecules of all the oxidation-reduction enzymes are grouped together at the same spot, (*b*) that molecules of oxidising enzymes and dehydrogenases occur in pairs, every conceivable pair being represented. It is difficult to form a concrete picture of this state of affairs, but in our present state of ignorance of the nature of oxidising enzymes and their condition inside the cell we cannot exclude the possibility.

The analogy of aerobic oxidation systems is in favour of hydrogen carriers being involved. Two systems have been described in which carriers play a part in linking two oxidation-reduction enzymes: (1) oxygen—indophenoloxidase—cytochrome—dehydrogenases—substrates [Keilin, 1929] and (2) hydrogen peroxide—peroxidase—phenol—ascorbic acid—reducing systems [Szent-Györgyi, 1928]. Other systems (*e.g.* oxygen—yellow pigment—dehydrogenase co-enzyme—hexosemonophosphate [Warburg and Christian, 1932]) utilise a carrier, which is however autoxidisable, and only the reduction of which is catalytic.

Evidence that carriers are required for anaerobic oxidation-reduction reactions could be obtained by the isolation from cells of substances capable of linking pairs of cell-free dehydrogenases, but all the known natural substances which have been considered as respiratory carriers have proved inactive (except pyocyanine) and our own attempts at detecting such substances have so far been unsuccessful. The failure of these natural carriers to link dehydrogenases is due to various causes. Cytochrome, though reduced rapidly by most of the dehydrogenases, cannot be reoxidised by even the most positive dehydrogenase system. Warburg's yellow pigment is reduced only by the hexosemonophosphate system and is not reoxidisable except by oxygen. Except for the reduction of glutathione by glucose dehydrogenase, oxidised glutathione, dehydroascorbic acid, lactoflavin and ovoflavin are not reduced at an appreciable rate and the reduced forms are probably not reoxidised by any enzyme considered in this paper.

Considerations of potential show that, if carriers are involved in reactions of this type, then at least two such carriers must be present in certain cells. For instance, the two reactions succinate-nitrate (r_H range 15–>20) and hydrogen-pyruvate (r_H range 0–5), both of which are catalysed by intact *Bact. coli*, cannot be linked by the same carrier.

The kinetics of linked reactions.

The factors influencing the efficiency of a dye as a hydrogen carrier in linking two dehydrogenases may be classified under four headings:

(1) The potential of the dye.

(2) The relative activity of the two enzymes.

(3) The concentration of the dye.

(4) Certain factors of specificity in the relation between enzymes and indicators.

(1) There is a general empirical rule that the more negative an indicator is, the more slowly it is reduced and the more quickly its leuco-form is oxidised.

Moreover it is clear that the maximum rate of reaction will be obtained when the rate of reduction of the dye by the negative system and the rate of reoxidation of the leuco-dye by the positive system are roughly equal. Hence, if a series of indicators of different potentials is tested with a given pair of dehydrogenases, those lying near the more positive enzyme will be oxidised slowly, and those near the more negative reduced slowly, while somewhere between the two systems will be an indicator which is oxidised and reduced at comparable rates; this last will function most efficiently as a carrier. These points are illustrated in Figs. 1, 2 and 3.

(2) Exceptions to the above rule will be found when there exists a great disproportion between the activities of the reducing and oxidising enzymes. If the oxidising enzyme is present in only very small amount, then the rate of oxidation of the leuco-indicators all through the series will be correspondingly diminished, and the point at which there is a balance between the rate of reduction and the rate of oxidation will lie towards the more negative end of the scale. This is illustrated in the case of the reaction between hypoxanthine (very active xanthine oxidase) and fumarate (relatively inactive succinoxidase), where the optimum carrier lies at the level of the more negative system (see Fig. 3, curve *B*).

(3) In considering the concentration of indicator, it must be remembered that two concentrations, *viz.* those of the oxidised and reduced forms, have to be dealt with separately, as both are playing a part in the reaction. In the various experiments plotted in Figs. 4 and 5, the degree of reduction of the indicators concerned varied practically from 0 to 100 %. In the former case, the concentration of reduced dye, on which the oxidising enzyme acts, is practically *nil*, so one would expect the system to require a high total indicator concentration to make it work at maximum speed. This case is illustrated in Fig. 5, curve *D* (methyl viologen linking fumarate-hypoxanthine, where the dye was $<0{\cdot}5$ % reduced). In the other case the concentration of the oxidised form, on which the reducing enzyme acts, is again practically *nil*, so again the need for a high total concentration will be expected. This case is illustrated in Fig. 5, curve *C* (methylene blue linking fumarate-hypoxanthine, where the dye was $>99{\cdot}5$ % reduced). When, on the other hand, the extent of reduction lies between, say, 10 and 90 %, the concentration of both forms is of the same order as the total concentration, and both enzymes have a sufficient concentration to oxidise or reduce at a good speed, even when the total concentration of dye is very small.

The other factor which determines what concentration of indicator is required to give maximum velocity of reaction is obviously the affinities of the various enzymes for the dyes and leuco-dyes. Exact values for these affinities are not known, but it is evident from our results that on the whole the affinities of formic dehydrogenase for dyes and of nitratase for leuco-dyes are greater than those of xanthine oxidase and succinoxidase.

(4) The subject of specificity with regard to certain artificial indicators and biological oxidation-reduction carriers has already been discussed in the experimental section. We cannot at present give any explanation of the fact that while one indicator functions perfectly as a carrier, another of almost exactly the same potential does not function at all. In the case of the acidic (sulphonated) dyes, the reason cannot be toxicity, as Quastel [1931] has shown that basic dyes are in general much more toxic than acidic dyes to oxidising enzymes.

"Anaerobic potential" of cells.

If a series of indicators is micro-injected into cells or added to cell suspensions, it is found that there is a potential level below which indicators are not reduced [Needham and Needham, 1925]. Aerobically this level is fairly positive in potential, anaerobically fairly negative (in starfish eggs, for example, the values are −0·06 v. and −0·17 v. [Chambers *et al.*, 1933]); the two levels are called the aerobic and anaerobic potential respectively. Machlis and Green [1933] have demonstrated that the so-called "aerobic potential" is determined purely by kinetics and has no thermodynamic significance. The experiments described in this paper indicate that the "anaerobic potential" of cells must likewise be determined by kinetics. Suppose that methyl viologen is added to liver cells saturated with the substrates of the various dehydrogenase systems. Xanthine oxidase, formic dehydrogenase *etc.*, will reduce the dye to some extent, but more positive enzyme systems such as the lactic and succinic dehydrogenases will reoxidise the reduced methyl viologen. Whether the indicator will remain in the reduced or oxidised form will obviously be determined by the relative speeds of oxidation and reduction, and will not be directly connected with potential. Furthermore, it is possible to obtain two different values for the "anaerobic potential" of the same mixed enzyme system, merely by choosing two different indicators whose relative rates of oxidation and reduction differ. For instance, in one experiment with the system hypoxanthine-fumarate (Fig. 3, curve *B*), Nile blue was about 95 % reduced, indicating a potential of about −0·20 v., while benzyl viologen was 50 % reduced, indicating a potential of about −0·36 v. Obviously the ratio $\frac{\text{rate of oxidation by fumarate}}{\text{rate of reduction by hypoxanthine}}$ was not so much greater with benzyl viologen than with Nile blue as the difference in potential would lead us to expect.

Summary.

1. Dehydrogenases isolated from cells will not react with one another, unless an oxidation-reduction indicator is added.

2. The indicator functions by being continually reduced by the negative enzyme system and oxidised by the positive enzyme system.

3. None of the known constituents of cells, except pyocyanine, which can be oxidised and reduced (glutathione, cytochrome, ascorbic acid, Warburg's yellow pigment, lactoflavin, ovoflavin, citrinin and pyocyanine), is able to link any pair of dehydrogenases,

4. Some aspects of the kinetics of such "carrier-linked" reactions are described.

5. The bearing of these facts on the mechanism of anaerobic oxidations in intact cells is discussed.

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CCXLI. THE BIOLOGICAL AND TITRIMETRIC DETERMINATION OF VITAMIN C.

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The present communication deals with a comparison of the biological and titrimetric methods of determination of vitamin C, the materials examined being lemon juice, dried hips and ascorbic acid.

The biological determination of vitamin C.

The experiments were carried out by the protective method. Guinea-pigs, weighing about 200–250 g., were used, since these animals react very readily to vitamin C deficiency. The animals were put on a diet which was vitamin C-free but complete in other respects; to this diet a certain amount of the substance to be examined was added daily.

Working with batches of two or three animals, each of which received different additions, it was possible to determine the smallest quantity which just protected the guinea-pig from scurvy.

As the estimate is rather approximate in the absence of knowledge of the degree of latent scurvy in animals showing a negative gross result, it is important to ascertain the developmental degree of vitamin C-avitaminosis by microscopic examination of the root of the incisor of the guinea-pig before the animal shows any symptoms of scurvy.

Höjer [1924] proposed a method of estimation of vitamin C which depends on the fact that C-avitaminosis in guinea-pigs causes specific changes in the histological structure of the tooth manifested by degeneration of the odontoblasts with subsequent disturbance in the formation of dentine.

It is possible in longitudinal sections of the normal tooth to observe that the odontoblasts normally grow longer until fully developed and then atrophy and finally disappear.

Parallel with the development and degeneration of the odontoblasts runs the formation of the dental bone, the latter being fully developed when the odontoblasts in the crown of the tooth disappear.

In the case of C-avitaminosis the odontoblasts degenerate at an earlier stage, and with decreasing vitamin C supply the degeneration can be observed still closer to the root, finally occurring in the deeper part of the root itself.

Since the degeneration which the odontoblasts undergo in C-avitaminosis cannot be distinguished morphologically from the physiological degeneration, histological examination must be made of sections where normally only fully developed odontoblasts can be found, *i.e.* of sections through the fang part of the tooth.

According to Höjer the section ought to be taken horizontally on the lower jaw through the middle part of the front molar; this plane of sectioning is likely to hit the fang of the incisor horizontally also, and the measurement of vitamin C is then determined by the minimum amount which is just capable of preserving the normal histology of this particular region of the tooth.

A classification may also be carried out. Based on the histological conditions Key and Elphick [1931] have constructed a scale comprising five groups calculated for an experimental

period of 15 days starting from the picture corresponding with no protection (series 0) and ending with the normal (series IV). The experiments are carried out as protective experiments. To ensure that the guinea-pigs at the beginning of the experiment are without latent scurvy and at the same time without significant stores of vitamin C, the animals are fed for 3 weeks on Key and Elphick's vitamin C-free diet with a daily addition of 50 mg. dried hips, this diet having proved to provide exactly the sufficient amount of vitamin C.

Guinea-pigs prepared in such a way, and as far as possible of the same weight, are put on experiment for 14 days, being fed on the above-mentioned diet with additions chosen for the experiment, the histological examination is made as before stated in the topographical sections according to Höjer and conforming to the scale of Key and Elphick.

On the 14th day the animals are killed. The lower jaw is split between the incisors; the lower jaw halves are taken out and adhering muscular tissue is removed. The tissue is fixed during 8–10 days in Orth's fluid and washed in running tap-water for 24 hours. It is decalcified by treatment for 3 days with 20 parts 5 % nitric acid + 3 parts 3 % iron-alum. The tissue is then placed in 5 % potash-alum for 24 hours and washed in running water for 24 hours. It is finally dehydrated and paraffin sections are stained with alum-haematoxylin-eosin.

The results obtained with dried hips, the juice of Messina lemons and ascorbic acid are shown in Table I. It was found that 1·2–1·5 ml. lemon juice, 50 mg. dried hips and 0·7 mg. ascorbic acid are equivalent, *i.e.* they have the same biological effect since these doses are able just to preserve the normal structure in Höjer's section. Lemon juice accordingly contains 0·4–0·6 mg. ascorbic acid per ml. and dried hips contain 1·4 % ascorbic acid.

Table I. *Showing degree of protection against scurvy, measured on the Key and Elphick scale, afforded by various substances.*

Preparation	mg.	Key and Elphick scale	Preparation	ml.	Key and Elphick scale
Ascorbic acid	0·6	III–IV	Lemon juice	1·5	III
,,	0·6	III	,,	1·5	IV
,,	0·9	IV	,,	2·0	IV
,,	0·9	IV	,,	2·0	IV
,,	1·2	IV	,,	2·5	IV
,,	1·2	IV	,,	1·0	III–IV
,,	0·6	III	,,	1·0	III–IV
,,	0·6	III	,,	1·5	IV
,,	0·6	II–III	,,	1·5	IV
,,	0·9	IV	,,	2·0	IV
,,	0·9	IV			
,,	0·9	IV		mg.	
,,	0·64	II–IV	Dried hips	30	II–III
,,	0·64	III	,,	30	II–III
,,	0·8	IV	,,	40	III
,,	0·8	IV	,,	40	III
,,	0·64	IV	,,	50	IV
,,	0·64	III–IV	,,	50	IV
,,	0·8	IV	,,	60	IV
,,	0·8	IV	,,	60	IV
			,,	80	IV
			,,	80	IV

Titrimetric determination of vitamin C.

To determine vitamin C titrimetrically 2 : 6-dichlorophenolindophenol can be used as combined oxidant and indicator [Tillmans *et al.*, 1932]. These workers suggest the use of a solution of 0·001 *M* 2 : 6-dichlorophenolindophenol at a reaction of p_H 5–6. By this method an intense blue end-point is secured, while a weaker purple end-point is obtained at a more strongly acid reaction. The latter end-point is utilised by Harris and Ray [1933] in order to circumvent

the danger of other reducing substances influencing the reaction. Harris and Ray also suggest an "inverse" titration, *i.e.* admixture of the solution to be tested with a previously measured 0·01 *M* indicator solution.

We preferred after a thorough trial to follow Tillmans's directions, using however a somewhat stronger indicator solution. With the relatively concentrated ascorbic acid solutions obtained from hip extracts and lemon juice a considerably stronger indicator concentration than 0·001 *M* can be used with advantage. To neutralise free acid 50 % sodium acetate was added. The destruction of the indicator itself at a too strongly acid reaction is in favour of the above-mentioned technique. At alkaline reactions the reduction takes place too slowly and the vitamin C is too easily oxidised by the oxygen of the atmosphere; this error can be avoided by working at p_H 5–6. Furthermore it is advantageous to employ the easily discernible blue end-point.

The "inverse" titration of Harris and Ray can only be used with stronger ascorbic acid solutions, because the determination of the moment when the colour disappears becomes difficult at high dilutions. It has been found to be more certain to determine the appearance of a colour than its disappearance, and therefore the usual method of titration was preserved. At the indicated p_H the titration can be carried out without running the risk of autoxidation of the ascorbic acid but for additional security we avoided too strong daylight and did the titration as fast as possible. The ascorbic acid used for the purpose was a pure preparation, placed at our disposal by Prof. Szent-Györgyi. Its purity was confirmed by iodimetric titration in our laboratory to be 100 %.

Hip extract. To obtain this extract of hips a small amount of dried hip-powder (produced by the Vitamin Laboratory, Skelskor) was boiled for 10 minutes under a vigorous stream of nitrogen in a proper amount of water, a few drops of sulphuric acid being added. The loss of water by evaporation was made good. After refrigeration the mixture was filtered *in vacuo* from a separating-funnel from which the air had been displaced with nitrogen or carbon dioxide. The separating-funnel was closed by a glass stopper and its stem passed through a tight-fitting rubber stopper to discharge into a conical filter-funnel which was contained in a previously evacuated desiccator. An aliquot of the filtered solution was then titrated with 2 : 6-dichlorophenolindophenol with addition of sufficient sodium acetate to produce a blue end-point.

For 0·345 g. hip-powder were used 80 ml. water. 10 ml. were titrated and reduced 1·45 ml. 2 : 6-dichlorophenolindophenol, of which 2·80 ml. had been ascertained to be equivalent to 1 mg. ascorbic acid.

It thus follows that 0·345 g. of hips is equivalent to $8 \times 1{\cdot}45$ ml. $= 11{\cdot}60$ ml. indicator solution supposing all vitamin C has been extracted. Consequently 12·0 mg. or 1·20 % ascorbic acid were contained in 1 g. hips.

After careful filtration a second boiling-out by the same method was performed, but the filtrate therefrom appeared to be incapable of reducing the indicator solution.

From time to time similar extractions of hip-powder have subsequently been made. The second extraction never gave any titration value; it is safe to assume therefore that all vitamin C was removed by the first extraction. It must however be noted, that it is somewhat difficult to obtain constant values. The above-mentioned represents the highest value obtained.

There seem to be only two possible explanations of the variation: either an incomplete extraction of the hip-powder or a destruction of part of the vitamin C in spite of all precautions. It is reasonable then to take the highest attained value as representing the content of vitamin C in hip-powder obtained by titration,

supposing of course that no other substances capable of reducing 2 : 6-dichlorophenolindophenol are contained in the extract.

However, as is evident from the above-mentioned biological experiments, the actual content of ascorbic acid is somewhat higher than the maximum value obtained by titration; the latter must consequently be taken in practice to represent a minimum value.

Lemon juice. Titration of ascorbic acid in lemon juice was performed with two different shipments of lemons. The experiment with guinea-pigs lasted for 14 days and samples of the juice were titrated daily.

For each titration 15 ml. of juice were used; after expression 6 ml. 10 % normal lead acetate solution were added, followed by 9 ml. 20 % sodium sulphate to precipitate excess of lead.

After filtration, the perfectly clear solution was titrated with 2 : 6-dichlorophenolindophenol, which, as in the case of the previous titration of hip extract, had been standardised against pure ascorbic acid. The titration value was calculated on the basis of the original undiluted juice.

In all 14 titrations were carried out, which on an average gave a content of 0·39 mg. ascorbic acid in 1 ml. of lemon juice. This first experiment lasted from Nov. 29th to Dec. 13th, 1933, inclusive; in the second experiment from March 12th to 25th, 1934, inclusive the juice on each day was similarly titrated, and these titrations gave on the average 0·56 mg. ascorbic acid in 1 ml. of lemon juice.

It was evident that the juice used in this last experiment was considerably richer in vitamin C than the juice used in the preceding experiment. It was also evident from the appearance of the lemons that the latter were far riper than the lemons from the preceding experiment.

Summary.

It is evident from these results that for the guinea-pig 0·64 mg. ascorbic acid corresponds very nearly to the necessary addition of vitamin C to a diet deprived of all its vitamin C beforehand, while 0·8 mg. is thoroughly satisfactory. The true value is then to be found between these, *i.e.* about 0·7 mg.

Correspondingly the necessary amount of lemon juice in the first experiment lies between 1·5 and 2 ml., in the second experiment near to 1 ml. Approximating these values at 1·75 ml. and 1·25 ml. respectively we obtain 0·4 and 0·55 mg./ml. as the contents of ascorbic acid in the juices.

For dried hips the necessary minimum lies between 40 and 50 mg. Taking this to be 45 mg. it is evident that dried hips contain 1·5 % ascorbic acid.

A comparison between the results of the biological determination and those found by titration shows fairly good concordance; only in the case of dried hips is a slightly higher content of ascorbic acid found by biological assay, probably because the preparation of the solution for titration fails to extract the whole of the ascorbic acid.

The analyses of the lemon juice have revealed considerable variation in the vitamin C content. Consequently lemon juice has to be considered unsuitable as a vitamin C standard.

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CCXLII. QUANTITATIVE STUDY OF THE THYROTROPIC ACTIVITY OF ANTERIOR PITUITARY EXTRACTS.

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The earliest experiments on hypophysectomy showed that atrophic changes take place in the thyroid after removal of the pituitary gland [Cushing, 1912; Dott, 1923]. This relationship is now well authenticated [Houssay *et al.*, 1931, for the dog; Smith, 1930, and Anderson and Collip, 1933, for the rat; White, 1933, for the rabbit]. Hypophysectomy also abolishes the power of the thyroid to undergo compensatory hypertrophy [Houssay *et al.*, 1932; Smith, 1930]. Replacement therapy has been successful in overcoming the effect of hypophysectomy. Thus Smith [1930] was able to restore the atrophied thyroid of the hypophysectomised rat by the implantation of anterior lobe tissue, while Anderson and Collip [1933] prevented atrophy by starting treatment immediately after hypophysectomy. Similar results have been obtained by Freud *et al.* [1933] for the rat, and Houssay *et al.* [1932] for the dog.

Normal animals have naturally been more used for studying the thyrotropic effect of anterior lobe extracts. As early as 1908, Rénon and Delille [1908, 1, 2] administered pituitary extracts to rabbits and observed changes which they considered as indicative of thyroid hyperactivity. Successful experiments on rabbits have also recently been reported by Baumann and Marine [1932]. It is now evident that the thyroids of different species vary greatly in their power to respond to the thyrotropic substance. The guinea-pig is particularly sensitive, and, as Freud *et al.* [1933] remark, has become the classical animal for the study of thyroid hyperplasia. The reaction in this species was obtained by Loeb and Bassett [1929; 1930] and Aron [1929], and afterwards by a large number of workers, including Janssen and Loeser [1931], Loeser [1932], Junkmann and Schoeller [1932], Anderson and Collip [1933], Castillo [1932], Greep [1933], and Friedgood [1934]. Marine and Rosen [1934] found that thyroid transplants can be stimulated as well as normal glands. Characteristic histological changes are produced in the guinea-pig thyroid. In the early stages of stimulation the epithelium undergoes hyperplasia, and becomes very high columnar, with large rounded nuclei. At the same time the colloid in the follicles becomes vacuolated and gradually decreases in amount. The gland may increase greatly in weight and macroscopically has a much more fleshy appearance than is normal. At the same time a rise occurs in the basal metabolism and symptoms of exophthalmos, presumably indicative of increased thyroxine output, may subsequently appear [Friedgood, 1934; Anderson and Collip, 1934].

In the rat however Loeb and Bassett [1929], Aron [1930, 2; 1932, 2], Schockaert [1932], and Anderson [1933] all failed to produce abnormal hyperplasia of the

thyroid of the rat by injection of anterior lobe extracts. Junkmann and Schoeller [1932] found the rat thyroid to be far less sensitive than that of the guinea-pig, while Houssay [1932], though obtaining a slight effect, states that a dose which will cause a response in a 10 kg. dog or in a guinea-pig is not adequate to affect a rat. Anderson and Collip [1933] however produced an effect in the rat and an increased metabolic rate with a purified preparation. These authors [1934] have now published a full account of their work on normal and hypophysectomised rats. Freud *et al.* [1933] obtained a slight effect on the rat.

The explanation of the comparative insensitivity of the thyroid of the rat to anterior lobe extracts seems to lie in the fact that the thyroid of this species is normally hyperactive. In this connection Junkmann and Schoeller's observation that the rat pituitary contains much more of the thyrotropic substance than that of the guinea-pig may be recalled. As Collip [1934] says, "it may follow as a general rule that the sensitiveness or responsiveness of an animal to injected hormone varies inversely with the hormone content or production of the animal's own gland," though this generalisation does not hold for the gonadotropic activity of the rat and rabbit pituitary as described by Hill [1934].

Various workers have shown that similar extracts of organs other than the anterior pituitary body do not affect the thyroid [*cf.* Aron, 1930, 2], and that no symptoms of hyperthyroidism are produced by administration of pituitary extracts to thyroidectomised animals [Verzár and Wahl, 1931]. It may thus be regarded as established without doubt that the anterior pituitary body elaborates a specific substance which stimulates the thyroid gland and which is presumably concerned with the regulation of thyroid activity in the normal animal.

In spite of the large amount of work which has been done on the thyrotropic activity of anterior pituitary extracts, remarkably little attention has been paid to the quantitative measurement of such activity, and the present paper is devoted to this aspect of the subject.

I. *Test animal and criterion of response.*

The work summarised above makes it evident that the guinea-pig is the obvious test animal for thyrotropic preparations. Possible criteria of response fall into two categories: (*a*) changes in the thyroid itself and (*b*) metabolic and other systemic changes depending on the increased production of thyroxine. The systemic changes, in addition to being extremely difficult to put on a quantitative basis, except perhaps the basal metabolic rate, suffer from the disadvantage that they involve two variables: (*a*) variation in the degree of thyroid stimulation produced by a given amount of anterior lobe extract and (*b*) variation in the secondary effect produced by a given amount of thyroxine. In this particular, of course, use of the B.M.R. or any other general effect is equivalent to the use of the vaginal or uterine reaction in assessing the gonadotropic activity of anterior lobe extract. Chief among workers using the B.M.R. is Collip, who defines a unit as "the minimum amount administered daily in two injections, which will cause a rise of 20 % in the metabolism of the hypophysectomised rat by the fourth day" [1934].

Direct examination of the stimulated gland is more satisfactory in some ways, and the changes in the size and histological appearance of the gland have often been used as test objects. Aron [1930, 1; 1932, 1], Loeser [1932] and Severinghaus [1933] give detailed descriptions of the histological criteria of stimulation, while Heyl [1933] considers the precautions necessary in defining stimulation histologically. Junkmann and Schoeller [1932] define a unit as the

amount required to produce a definite histological reaction in one out of two animals. The work of Castillo and Magdalena [1931] seems to throw some doubt on the validity of considering slight histological hyperplasia as a sign of extraneous stimulation, a view shared by Collip [1934]. Up to the present there appears to have been no systematic examination of the weight changes in the stimulated thyroid—*a priori*, an easy method of assay—and we have tentatively selected this criterion as a basis for the quantitative assay of thyrotropic preparations.

II. *Technique.*

Size of animal. Immature female guinea-pigs as near as possible to 200 g. body weight were used. The size and variability of the thyroid at this body weight are considered in the next section. All animals used were from the inbred strain maintained at the Institute's Farm Laboratories, the availability of which has made possible the work described in this paper.

Extract. One extract only (T.17.B) was used for the work described in Section III on the variables affecting the response of the thyroid. This was a dry friable powder obtained by precipitating with alcohol and ether a neutralised alkaline extract of acetone-desiccated ox pituitaries. The powder which represented about 10 % of the original desiccated gland or about 2 % of the fresh gland was readily soluble in dilute sodium hydroxide and stayed in solution when neutralised for injection. All injections were subcutaneous.

Details of other extracts used are given in the relevant places.

Weighing and histology. The thyroids, which can be dissected out very cleanly in the guinea-pig, were removed, fixed in Bouin's fluid and weighed at the 70 % alcohol stage in the up-grading. Certain of the glands were sectioned at 7μ and stained in the ordinary way.

III. *Examination of variables in the assay of thyrotropic activity on the immature guinea-pig.*

This section is concerned with an examination of the initial variability of the thyroid of the immature guinea-pig, and the variability in response to anterior lobe extract, together with a consideration of the most efficient system of injection *etc.* All the experiments described in this section were carried out with a single preparation, T.17.B, which under the conditions of preservation showed no signs of deterioration.

(*a*) *Initial size of the thyroid.* Since it would be extremely difficult to secure groups of young guinea-pigs of exactly the same body weight, and since even then variability in size of the thyroid might be expected, we have investigated the size and variability of the thyroid of normal guinea-pigs over a range of body weight more than covering all the animals used in these experiments. Thirty-eight normal animals with body weights of 150–300 g. were examined. The weights of the thyroids, plotted against body weight, are shown in Fig. 1. As will be seen, the mean thyroid weight at 200 g. body weight is 31 mg. The positions of these points obviously indicate a linear relation over the range of body weight used. Dealt with as a correlation table, these figures show a correlation coefficient of 0·808. The regression line calculated for these points has the formula $y=0{\cdot}159x-1{\cdot}02$, in other words the thyroids increase in weight almost exactly 1 mg. for each 6 g. increase in body weight. Individual variation is slight. Actually, even including the increase due to increasing body weight, the average thyroid weight for these 38 animals, 34·6 mg., only has a standard error of $\pm 1{\cdot}3$. It may be concluded therefore that both the size changes due

to increasing body weight, and also the individual variation in thyroid weight, in animals of 150–300 g. body weight, are insignificant compared with changes of the magnitude of those described below. Even so however we have made every effort to obtain animals of about 200 g. at the beginning of the experiment, and where the experiment was of sufficiently long duration to allow of appreciable body growth, we have taken into consideration the normal growth which would have taken place in the thyroids. The subsequent growth of the normal guinea-pig thyroid, as well as that of the adrenals and reproductive organs of both sexes will be dealt with elsewhere.

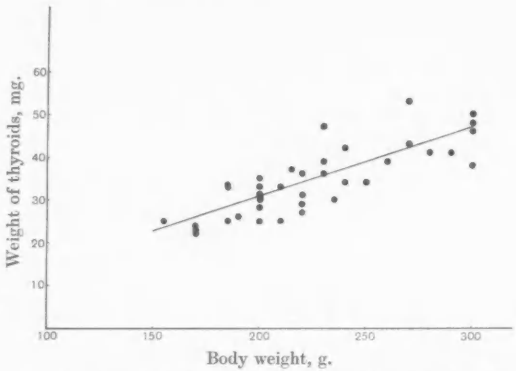

Fig. 1. Weight of the thyroids of the guinea-pig of 150–300 g. body weight.

Table I. *Effect of duration of injection.*

Daily amount given mg.	Days injected	Body weight when killed g.	Thyroid weight mg.	Normal thyroid weight for the same body weight mg.	Increase in weight of thyroids above normal mg.
2	3	214	45·2	33·0	12·2
,,	5	211	53·8	32·5	21·3
,,	8	207	60·2	31·9	28·3
,,	10	248	62·8	38·4	24·4
,,	14	208	60·0	32·0	28·0
5	2	198	47·0	30·5	16·5
,,	5	187	58·0	28·7	29·3
,,	6	170	61·2	26·0	35·2
,,	10	188	78·2	28·9	49·3
,,	15	220	81·0	34·0	47·0
,,	20	254	84·2	39·4	44·8

Five animals per group, except in the first 5-day group, which had ten, and second 5-day group which only had four.

(*b*) *Effect of duration of injection.* Our first experiment was designed to show what was the most suitable period of injection for securing a good reaction without unduly prolonging the time of injection. Five groups of animals were therefore injected with 2 mg. daily of the preparation, groups being killed after 3, 5, 8, 10 and 14 days of injection. The results are shown in Table I. Measured either by the gross weight of the thyroids, or by their increase above normal for the body weight of the group, the maximum reaction is reached at 8 days

and further daily injection does not increase the response. A further six groups were given 5 mg. daily of the same preparation for 2, 5, 6, 10, 15 and 20 days; each group being killed on the day following the last injection. As with the smaller dose, the maximum thyroid weight is reached quite soon, in this case at 10 days, and further injection of the same daily amount hardly increases the response. On the other hand, there is no sign of any failure to maintain the development induced by 10 days of injection. So far as these results go, they do not suggest that insensitivity and failure to maintain early hypertrophy appear in the course of prolonged injection, as described by Collip and Anderson [1934]. It is possible, of course, that 20 days is inadequate for the development of what they term an "anti-hormone" phenomenon, but according to Anderson and Collip [1934] the increased B.M.R. begins to fall after 7 days' injection.

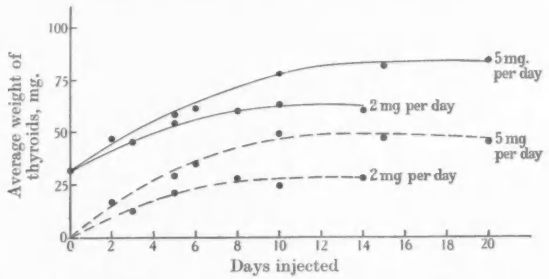

Fig. 2. Effect of duration of injection of anterior lobe extract on the weight of the thyroid.
——— Gross weight of thyroids.
– – – – Increase above normal for the corresponding body weight.

The maximum response with 2 mg. daily, reached at 8 days, was a thyroid weight of 60 mg., about double the normal for the body weight. With 5 mg. daily the maximum was about 80 mg., reached at 10 days, and the curve was higher from the beginning. The maximum development attained with 5 mg. daily seems to come about 2 days later than that with the smaller dose. In both these series most of the growth took place during the first 5 days, and this time was obviously adequate for producing a good reaction. As there is little to be gained by continuing beyond 5 days, we have adopted this as a standard period of injection.

(*c*) *Time of killing after last injection.* It was unlikely that there would be much delay in the response to treatment, so the animals described above were killed on the day after the last injection. It seemed desirable however to investigate this point and at the same time to extend the observations to ascertain whether the hypertrophied gland underwent regression and, if so, at what rate. Groups of animals were given 10 mg. Extr. T.17.B daily for 5 days, an amount known to produce marked hypertrophy of the thyroid. The groups were killed at 1, 5, 10 and 20 days after the last injection. Results are shown in Table II, and Fig. 3. Since the body weight increased appreciably in animals killed in the later stages, a more accurate value for the extent of the regression is obtained by considering the excess thyroid weight above normal for the size of the animal. On this criterion, the weight of the hypertrophied thyroid drops rapidly in the first few days after the cessation of injection until about half the excess weight is lost. Subsequent regression is slow so that 20 days after the last injection

the thyroid is still slightly above its proper weight. The thyroid presumably regains its normal relation to body weight partly as a result of its own regression and partly as a result of body growth. These findings make it clear that to measure the maximum hypertrophy of the thyroids the animals should be killed very soon after the last injection, and as a routine we have killed on the following day.

Table II. *Involution of the hypertrophied thyroid after the cessation of injection of anterior lobe extract.*

Time killed after last injection days	Body weight when killed g.	Thyroid weight mg.	Normal thyroid weight for same body weight mg.	Increase in thyroid weight above normal mg.
1	217	82·6	33·6	49·0
5	242	62·4	37·5	24·9
10	264	57·8	41·0	16·8
20	289	51·8	44·9	6·9

Ten animals in the first group, five in the others.

Fig. 3. Involution of the hypertrophied thyroid after the cessation of injection of anterior lobe extract.
——— Gross weight of thyroids.
- - - - - Excess above normal for the corresponding body weight.

(*d*) *Effect of various doses over a constant period.* After determining the growth and variability of the normal thyroid, a suitable duration of treatment and the time of killing after the last injection, it was necessary to consider the response in relation to dosage, so that a standardisation curve could be made. In the first experiment a wide range of dosage was used. Five groups of five animals each were injected with 4, 10, 20, 30 and 40 mg. of Extr. T.17.B daily for 5 days. The results (Table VII and Fig. 6) showed that 10 mg. daily caused enlargement to 82·6 mg. and that further increase of dosage had little effect. The group receiving 40 mg. daily actually had slightly smaller thyroids than the one receiving 30 mg., but this may have been due in part to their having a slightly lower body weight. In view of these results it was clear that hypertrophy above 80 mg. weight was no use for assay work but that the steepness of the response/dose curve during rapid enlargement of the thyroid offered good chances of obtaining a curve suitable for assay work.

Attention was next turned to extending the experiments on hypertrophy up to 80 mg. Groups of ten guinea-pigs were injected with 2 and 7 mg. daily, and the previous experiments were duplicated so as to bring the groups at 4 and 10 mg. daily up to ten animals. The final results for this dose/response curve are shown in Table III and Fig. 4. The control figure was obtained from

a group of ten uninjected animals of comparable body weight. The curve given by these results is of a well-recognised type and can obviously be used as the basis of a method of assay. The statistical aspect of the use of this curve for this purpose is discussed in Section IV.

Table III. *Relation between dose given and weight of thyroid produced.*

Amount given daily mg.	Body weight when killed g.	Thyroid weight mg.
0	208	31·8
2	211	53·8
4	210	64·2
7	202	79·9
10	217	82·6

Ten animals per group.

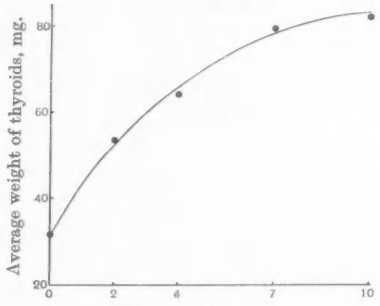

Fig. 4. Relation between dose of anterior lobe extract given and weight of thyroids.

(*e*) *Effect of a constant total dose over various periods.* From the data discussed above it is possible to obtain information (summarised in Table IV) of the effect

Table IV. *Effect of duration of administration of a constant total dose.*

Total amount given mg.	Amount daily mg.	Days given	Thyroid weight mg.
10	2	5	53·8
	5	2	47·0
20	2	10	62·8
	4	5	64·2
50	5	10	78·2
	10	5	82·6

Five or ten animals per group.

of varying the time over which a constant total dose is given. It is evident that where the period of injection is not extended unduly, the influence is surprisingly slight: thus 10 mg. Extr. T.17.B give much the same effect whether given as 2 mg. for 5 days or as 5 mg. for 2 days, while 5 mg. for 10 days give a very similar result to 10 mg. for 5 days. It is evident from Fig. 2 however that where injection is carried on beyond 10 days the efficiency of a constant total dose will fall. Thus 2 mg. for 20 days would presumably not give a thyroid weight much above 60 mg., whereas from Fig. 4, 8 mg. for 5 days would give a weight of nearly 80 mg.

(*f*) *Control experiments.* A control experiment on the effect of injecting foreign material was made by giving a similarly prepared extract of rat testis in comparable amounts for 5 days. As will be seen from Table V there was no

Table V. *Control experiments.*

Substance given	Total amount	Body weight g.	Thyroid weight mg.	Ovary weight mg.	Uterus weight mg.
Alkaline extract rat testis	From 5 g.	202	27·4	—	—
Urine of pregnancy extract	50 rabbit units	193	29·4	82·6	136·0
Oestriol	1·2 mg.	190	26·6	55·7	316·0
Anterior lobe extract to ovariectomised animals	From 5 g.	207	83·2	—	104·0
Anterior lobe extract	25 mg. T.17.B	186	60·0	29·0	101·4
Anterior lobe extract to ovariectomised animals	25 mg. T.17.B	192	51·2	—	101·0
Anterior lobe extract to ovariectomised animals receiving oestriol	25 mg. T.17.B + 1·2 mg. oestriol	186	40·4	—	328·4

Five animals per group.

result on the thyroids. Other control experiments were necessitated by Bialet-Laprida's [1933] conclusion that oestrin has an inhibiting action on the thyroid. Since the thyrotropic extract used by us contained the gonadotropic principles, which might be responsible for the secretion of oestrin by the immature ovary, it seemed necessary to ascertain what effect, if any, this complication might have on the response of the thyroid to the thyrotropic substance. In the first experiment, oestriol (trihydroxyoestrin) was given to a group of animals. As shown in Table V, this treatment caused a marked reaction in the uterus and the vulva became patent. The average thyroid weight, however, was only a little over 1 mg. below the normal for the body weight. This experiment, carried out with pure crystalline oestriol, fails to give any confirmation to Bialet-Laprida's statement that a comparatively small dose of folliculin will halve the weight of the immature thyroid. In the second experiment, 5 mg. daily for 5 days of a urine of pregnancy extract were injected into each of a group of animals. This dosage, in rabbit ovulation units, contained about the same gonadotropic activity as the largest of the doses of T.17.B given. The ovarian response was very strong and there was appreciable uterine reaction, indicating secretion of oestrin. The thyroid weight was entirely normal.

These experiments however did not necessarily preclude the possibility of the presence of oestrin inhibiting thyroid hypertrophy as suggested by Aron and Benoit [1932]. In other experiments, therefore, alkaline extracts of two samples of the same batch of dried gland, made in the same way, were injected in similar amounts into (*a*) normal immature guinea-pigs, (*b*) ovariectomised immature guinea-pigs. The average thyroid weights for the two groups happened to be identical (83·2 mg.) and there is no doubt that the thyrotropic activity observed in the two groups was very similar, and that any stimulation of oestrin production in the first group had not materially affected the thyroid response.

Finally, 5 mg. daily of T.17.B were injected into groups of (*a*) normal immature guinea-pigs, (*b*) ovariectomised animals and (*c*) ovariectomised animals simultaneously injected with 0·24 mg. oestriol daily. The results (Table V) show that large doses of oestrin tend to inhibit thyroid hypertrophy. The thyroids of

the normal animals receiving the anterior lobe extract however were appreciably heavier than those of the ovariectomised animals, and it seems certain that the amount of oestrin produced by the ovary under the influence of pituitary extracts such as these can have had no inhibiting effect on the thyroid development. In actual fact the amount of oestrin produced in most of the test animals (as judged by the size of the uterus and by the failure of the vulva to become patent) seems to have been remarkably small, a conclusion in keeping with Hill's [1934] observation of the low activity of ox pituitary tissue on immature animals.

IV. *Standardisation curve for thyrotropic substance and definition of a unit.*

Prof. Gaddum pointed out to us that the curve shown in Fig. 4 is of the type which placed on a logarithmic co-ordinate becomes a straight line, and that in such a form it is more suitable for use in assay work. Conversion to a logarithmic scale necessitates omitting the point for the control animal (dosage 0). The other four points of Fig. 4 are shown on a logarithmic scale in Fig. 5, from which it is evident that they fall on a good approximation to a straight line. The line is so obvious that there seemed little to be gained by calculating its regression formula.

It must be remembered that assay curves of this kind change both in position and in slope, and that if tests are made only infrequently it may be necessary to give two doses of a standard and two doses of the unknown extract for each determination. At present we have no information as to the probable changes in the curve from time to time. The whole of the work described in this paper was carried out over a comparatively short space of time, the standardisation curve being made simultaneously. The groups of ten animals used for the standardisation curve are actually composed of groups of five done at different times (3–4 weeks apart), and in only one was there appreciable difference in the results obtained from the paired groups of five animals. This means little since the time range was short, but as a basis for comparing the results described elsewhere in the paper we have considered the standardisation curve shown in Fig. 5 as adequate. It is evident however that steps would have to be taken to verify the slope and position of this curve before it could be used in future work.

At present we are concerned therefore only with the inherent error due to individual variation in response. It is possible to consider this by the usual methods.

Each of the four points is the mean figure for ten animals; and σ is 9·0, 7·5, 15·9 and 11·3 for the 2, 4, 7 and 10 mg. daily means respectively. The high figure for the third point was mainly due to a very eccentric figure for one animal, so that there is no indication here of a regular increase in variability as the response increases. The coefficient of variation (σ/mean $\times$ 100) is much less for the last group than for the first, so that the hypertrophied thyroid shows a lower percentage variation than the normal, though actual variability may be slightly greater. From this it seems reasonable to conclude that the hypertrophy caused by a given dose of anterior lobe extract tends to be a regular amount, and is not proportional to the original weight of the thyroid—a useful fact in view of the inevitable variation in the initial size of the gland.

In view of the above it seems justifiable to arrive at a σ for the 40 observations combined, which would give a σ for the thyroid response over the range of dosage and under the existing conditions. The figure obtained is 10·8, so that the standard error of the response would be $\pm$4·8, $\pm$3·4 and $\pm$2·4 respectively, using groups of 5, 10 and 20 animals. Turning again to the line in Fig. 5, it can

be found graphically that where u is the weight of the thyroid in mg. and x is the log of the daily dose of anterior lobe extract in mg., $du/dx = 47/1$. With a group of five animals therefore the error $\pm 4{\cdot}8$ in the determination of the mean will represent $\pm 4{\cdot}8/47 = 0{\cdot}102$ as the error in the determination of the log of the dose given. This is equivalent to an error of about 25 % in the determination of the dose, and also therefore in the determination of the unit as shown on the top co-ordinate in Fig. 5. For groups of ten animals, the error is reduced to a little above 15 %; with groups of twenty animals, the corresponding figure is near 10 %.

It is clearly not worth doubling the cost and labour of the tests to reduce the error from 15 to 10 %, but groups of ten seem to be desirable. In practice we have generally been unable to use larger groups than five, which implies an error of ± 25 % in the results described below.

Adoption of a unit. Pending an international agreement as to a unit of thyrotropic activity, it is necessary to have some provisional definition for comparative work in the laboratory, and the size changes considered above seem to offer a suitable basis. It is evident that for purposes of practical convenience the unit should fall somewhere in the middle of the line shown in Fig. 5. The weights of the thyroids in the immature guinea-pig of the size we have used average very near 30 mg.; double this weight falls well into the most serviceable part of the dose/response curve. It seems sound therefore to name 60 mg. as the critical weight required, and to adopt as unit: the thyrotropic activity contained in an amount of extract, which, given daily for 5 days, will cause the thyroids of the immature guinea-pig to attain a weight of 60 mg. To cause such a response requires 3·0 mg. daily of the extract used for the experiments recorded in Fig. 5. The dosage shown on the bottom co-ordinate can be converted into units as shown on the top co-ordinate in the ordinary way. The unit so defined is obviously a large amount considered in terms of original pituitary tissue. Cow pituitary, for instance, contains only about 6 units per g. fresh tissue (or about 30 units per g. desiccated tissue). The adoption of a system of assay entailing a large unit has certain disadvantages, notably the larger consumption of material in assay work and the apparent decrease in the activity of extracts. There are however compensating advantages, particularly the fact that the diagnostic reaction is far removed from the range of normal variation.

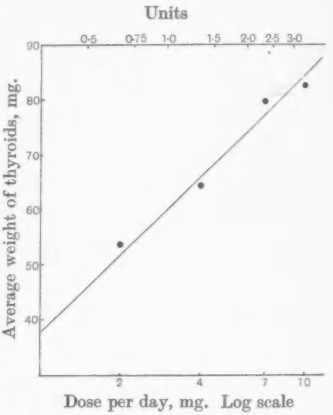

Fig. 5. Standardisation curve for thyrotropic hormone.

V. *Quantitative observations on the preparation of thyrotropic extracts.*

Advantage has been taken of the quantitative work described above to examine the efficiency of some of the processes commonly used in the preparation of thyrotropic extracts.

(*a*) *Acetone desiccation.* The initial acetone desiccation of the fresh gland is so useful a process and so widely used that it is important to know whether it entails loss of thyrotropic activity. 120 g. fresh ox anterior lobe were divided

into two portions. Part (*a*) was extracted overnight with 60 ml. *N*/10 NaOH, neutralised, centrifuged and the whole of the supernatant fluid injected into five animals over 5 days. Part (*b*) was desiccated in 500+500 ml. of acetone, dried and extracted overnight with 60 ml. *N*/10 NaOH+48 ml. water, this being the calculated amount of water removed in the desiccation. The extraction was completed and injections made as with the first preparation. The thyroids of the animals receiving the fresh tissue extract averaged 90 mg. in weight, those of the other group 101 mg. The second group of animals were of rather high body weight (average 240 g.) compared with the others used in these experiments, and this probably accounts for the very high thyroid weight obtained, but it is obvious that the acetone-desiccated gland retains its full thyrotropic potency.

(*b*) *Alkaline extraction.* Extraction of the acetone-desiccated tissue with dilute alkali, precipitation of the neutralised extract with alcohol and drying of the precipitate produces a clean friable powder which contains the active substances of the anterior pituitary in a much reduced bulk and forms a good basis for further purification. It seems important therefore to know how thoroughly the active substances are removed from the dried gland by this method. 100 g. acetone-desiccated ox anterior lobe were extracted with 1000 ml. *N*/20 NaOH overnight, with vigorous stirring at the beginning and at the end. The mixture was then neutralised and centrifuged. The supernatant fluid was precipitated with 5 vols. of 96 % alcohol and 1 vol. of ether and the precipitate dried and weighed. The residue was re-extracted with 500 ml. *N*/20 NaOH in the same way. The residue from the second extraction was finally re-extracted with 500 ml. saline. The results of the assays are shown in Table VI, and suggest

Table VI. *Number of alkali extracts.*

No. of extract	Nature of extract	Total extract obtained g.	Thyroid weight produced by 7 mg. daily mg.	Weight of unit mg.	Total units in extract (approx.)
T.19.B1	1000 ml. *N*/20 NaOH on 100 g. desiccated ox anterior lobe	10·05	71·0	4	2500
T.19.B2	500 ml. *N*/20 NaOH on residue of above	3·21	51·4	11	290
T.19.B3	500 ml. saline on residue of 19.B2	0·50	81·0	2½	200

7 mg. daily of each fraction given for 5 days to groups of five animals.

that so far as thyrotropic activity is concerned, though the second extraction may be worth while, the third is not. This relation, of course, might not hold in successive extractions of horse pituitary, in which the total soluble matter is much greater. The comparatively high activity of the small amount of material obtained by the third extraction is probably due to the removal of less inert matter by the saline than by the neutralised alkali. We have not yet assayed these successive fractions for gonadotropic activity, and such assay may, of course, modify the conclusion about the optimum number of extracts where maximum all-round activity is desired.

It may be noted here that the amount of dry extract given by the above method is of the order of 10–13 % of the original dried tissue.

(*c*) *Precipitation by alcohol.* Unless an extract is to be used immediately, it is highly desirable to reduce it to a dry powder. In the case of the alcohol-insoluble principles of the anterior pituitary, precipitation by alcohol is the

usual method for doing this, and it is generally assumed that the process is efficient. As we are not aware that there have been any quantitative experiments in relation to the thyrotropic activity, an experiment was performed in which the effect of injecting the neutralised alkaline extract was compared with the effect of giving the alcohol-ether precipitate from a comparable amount of extract. The average thyroid weights of the two groups of animals were less than 4 mg. different on a strong response, and precipitation by four volumes of alcohol and one of ether therefore seems a safe procedure.

(*d*) *Solubility in pyridine.* 50 % aqueous pyridine has been shown to be a most useful solvent for the gonadotropic substances [Fevold *et al.*, 1931]. Greep's conclusion [1933] that the thyroid hypertrophy is produced either by the luteinising principle or by another substance associated with it during preparation, shows that the thyrotropic substance, of whose separate identity there can be little doubt, is probably soluble in aqueous pyridine. Tests were made on three residues after aqueous pyridine extraction of dried ox anterior lobe. In the first preparation only one pyridine extraction was made. Assay of the residue showed that about 4 units of the thyrotropic substance remained. In the second experiment, 20 g. dried gland were extracted four times with 500 ml. lots of aqueous pyridine. A residue of 15·7 g. was obtained; tests on this showed that only about 2 units per g. remained. In the other experiment, 100 g. dried gland were extracted twice with 2·5 l. of 50 % aqueous pyridine. The residue (T.14.A), when dried, weighed 45 g. and contained a little over 2 units per g. As opposed to this, over 500 units were recovered from the pyridine extract in spite of the heavy loss which may have occurred during evaporation. These experiments make it clear that pyridine extraction either removes or destroys the thyrotropic activity. The latter alternative is disposed of by the next experiment.

The evaporation of large amounts of pyridine extract is extremely troublesome, and it seemed that it might be much more convenient not to make the pyridine extraction on the large bulk of desiccated gland but to reserve it until the bulk had been reduced by preliminary alkaline extraction and alcohol precipitation. In practice, this procedure proved very satisfactory. 6·5 g. alcohol precipitate from the alkali extract of 50 g. dried gland, known to contain about 1500 units of thyrotropic activity (5 mg. daily = 64·2 mg. thyroids = 1·15 units), were extracted three times with a total of 200 ml. aqueous pyridine. The residue (T.18.C), washed and dried, weighed 2·9 g., and contained the barest trace, if any, of thyrotropic activity; 3 mg. daily for 5 days gave an average thyroid weight of 33·6 mg. The pyridine extract (T.18.D) was precipitated with alcohol and ether, and the precipitate, washed and dried, weighed 2·8 g. 3 mg. daily produced an average thyroid weight of 69·6 mg., an effect equivalent to 1·6 units. The unit weight of this fraction was therefore 1·9 mg., *i.e.* the total activity of this pyridine-soluble fraction was a little over 1450 units. This experiment seems to show conclusively that the thyrotropic substance is quantitatively removed by pyridine extraction and that a considerable purification is thereby achieved.

(*e*) *Separation from gonadotropic substances and prolactin.* In view of the solubility of the gonadotropic substances in pyridine[1] it is improbable that the above-described solubility of thyrotropic substance in pyridine will be of any use in separating these different principles.

[1] The results on the immature guinea-pig receiving T.18.C and D are rather confusing as regards the partition of gonadotropic activity. The ovaries of those receiving the pyridine-insoluble averaged 61 mg., more than twice the weight of those receiving the pyridine-soluble fraction. The uteri weights were the reverse.

On the other hand, interesting results have been obtained with the lactation-stimulating substance. The pyridine-extracted residue (T.14.A), which contained very small amounts only of thyrotropic activity, was tested on oestrin-sensitised immature rabbits, intact and ovariectomised and consistently gave a strong secretory proliferation of the mammary gland. A similar result was obtained with another pyridine-extracted residue tested on the mammary gland of intact or ovariectomised first oestrus rabbits. This result not only indicates that the thyrotropic and lactation-stimulating substances are distinct (as already shown by Riddle *et al.* [1933], but that they can be separated by pyridine extraction. Since Evans [1933] states that pyridine extraction usually leaves the growth hormone behind, it seems likely that a clean and simple separation of the growth hormone and prolactin from the thyrotropic and the gonadotropic substances may be effected by pyridine extraction.

(*f*) *Chemical properties.* In addition to what can be gathered of the chemical properties of the thyrotropic substance from the experiments described above, we are able to add qualitative data on certain other chemical properties. The substance is highly stable in the dry form. Ox pituitary tissue which had been desiccated more than a year was found to be active up to the usual standard. It is also stable to heat in the dry state. 7 mg. daily of a sample of T.17.B which had been heated to 100° *in vacuo* produced an average thyroid weight of 68·4 mg., which, though rather lower than that given by the unheated material, is not sufficiently different to suggest appreciable destruction. Heated to 100° in aqueous solution a similar dose gave an average thyroid weight of only 31·4 mg., which indicates total destruction of the thyrotropic principle. This latter finding is in keeping with what is usually assumed, but is contrary to the work of Krichesky [1934]. The substance is readily soluble in water. The pyridine extract of T.14 (see p. 1840) was extracted with 500+250+200 ml. of water. The insoluble fraction (T.14.B), weighing 2·9 g., gave a negative assay, while the water-soluble fraction (T.14.C), weighing 5·1 g., contained more than 500 units.

VI. *Simultaneous effects of total anterior lobe extracts on various organs.*

In addition to the weight of the thyroids, the weights of ovaries, uterus and adrenals were taken for all the animals described above. These records are of little interest here, except for those of the dose/response experiment (p. 1834). The average weight of the ovary at 200 g. body weight is about 30 mg. The highest average ovary weight for a group (82·5 mg.) was obtained from the urine of pregnancy experiment (p. 1836). Few of the anterior lobe extracts produced ovaries of more than 60 mg. The average uterus size for the group

Table VII. *Effect of various doses of anterior lobe extract on the organs of the guinea-pig.*

Daily dose mg.	Body weight g.	Thyroid weight mg.	Ovary weight mg.	Uterus weight mg.	Adrenal weight mg.
0	203	31·8	45·4	80·4	56·6
4	200	63·2	28·8	104·0	62·5
10	204	83·0	35·7	182·0	63·8
20	192	85·3	39·4	247·0	70·1
30	208	89·8	49·6	198·2	76·3
40	173	77·0	43·0	202·4	75·9

Five animals per group.

was extremely variable, ranging from 84 mg. to 202 mg. in 5-day experiments. Variation in adrenal size was comparatively slight though fairly regular and gave no suggestion that the normal immature guinea-pig would serve as a test animal for the adrenotropic hormone on gross size changes alone.

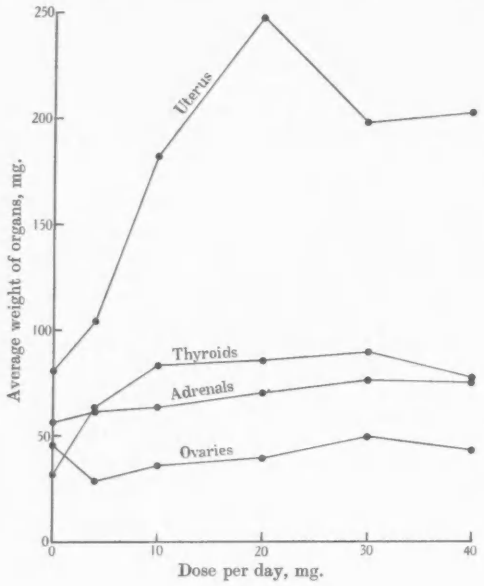

Fig. 6.

Table VII and Fig. 6 show the responses of the various organs to a wide range of dosage of T.17.B. The regularity of the responses of the thyroids and uterus is in marked contrast to the irregularity of the ovarian response.

VII. Summary.

1. The thyrotropic activity of anterior pituitary extracts was tested by their capacity to increase the weight of the thyroids of immature female guinea-pigs. At 200 g. body weight, the normal thyroids weigh about 31 mg. and are growing at the rate of 1 mg. per 6 g. body weight.

2. The maximum increase produced by a constant daily dose of anterior lobe extract is attained in 8–10 days. The standard period of injection in these experiments was 5 days, the largest thyroids produced in this time having an average weight of 101 mg. in a group of five animals.

3. Thyroids caused to enlarge to 80 mg. return to normal size about 3 weeks after the last injection.

4. A curve for response/dose has been made on groups of ten animals for the purpose of standardising extracts, and, pending some international agreement, the unit adopted for the laboratory is the thyrotropic activity contained in an amount of extract which, given daily for 5 days, will cause the thyroids of the 200 g. guinea-pig to attain a weight of 60 mg., *i.e.* about double the normal.

5. The efficiency of some of the methods commonly used in the preparation of thyrotropic extracts is examined in the light of these quantitative observations.

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CCXLIII. MANNOSEMONOPHOSPHATE. II. THE FERMENTATION OF MANNOSE BY DRIED YEAST.

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The isolation of mannosemonophosphate from the products of fermentation of glucose and fructose by yeast juice has been described in a previous communication [Robison, 1932]. Three components of the complex hexosemonophosphate of fermentation have now been identified as the monophosphoric esters of glucose, fructose and mannose; a fourth ester of low reducing power is present but has not yet been separated in pure condition. The disaccharide ester, trehalosemonophosphate [Robison and Morgan, 1928], may also occur in varying proportions among these products.

From the properties of the mannose ester and particularly from the identity of its osazone with that obtained from glucose- or fructose-monophosphate, it was inferred that the phosphoric acid group occupies the same position in all three compounds and that the new ester is therefore mannose-6-phosphate.

The experiments described in this and subsequent papers were planned to obtain further evidence as to the homogeneity and character of this substance and particularly to study the fermentation and esterification of mannose, in order to discover whether this sugar would yield a mixture of monophosphoric esters similar to that obtained from glucose and fructose. An answer to this question might help us to decide whether phosphorylation is preceded by a change in the hexose molecule or is itself the first stage in the fermentation process.

It was shown by Slator [1908] that mannose is fermented by almost all yeasts that ferment glucose but that the relative rates of fermentation vary with the type of yeast and especially with the treatment which the yeast has undergone. Yeast which is kept for some time or is heated loses its power to ferment mannose to a much greater extent than its power to ferment glucose. On the other hand, glucose and fructose are fermented under all conditions at approximately equal rates.

Harden and Young [1909] showed that mannose behaves towards yeast juice, both in the presence and the absence of added phosphates, in the same manner as glucose; the initial rates of fermentation were similar and on the addition of phosphate a rapid rise in this rate occurred, the extra amounts of carbon dioxide and alcohol produced being equivalent to the phosphate added. The fermentative power of the juice, however, fell off more rapidly with mannose than with glucose or fructose.

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From the products of the fermentation of mannose Young [1909] isolated a hexosediphosphoric ester identical with that obtained from glucose and fructose and now known to be γ-fructose-1:6-diphosphate.

I.S. Neuberg and Ostendorf [1930] fermented mannose and glucose at 37° with fresh yeast in presence of toluene and phosphate. From mannose they obtained two to three times as much monophosphate as from glucose, the proportion of hexosediphosphate being correspondingly low. In one experiment no diphosphate at all was found. The monophosphate fractions obtained from mannose showed high dextrorotations ($[\alpha]_D$, +45°, +34° for the free acids) but had low phosphorus contents. It seems likely that they contained trehalosemonophosphate.

Our own experiments were carried out with dried yeast prepared from English mild ale top yeast. We compared the fermentation and esterification of mannose, glucose and fructose at different temperatures, varying also the concentration of sugar and the amount of added phosphate.

Experimental.

Method of experiment.

The dried yeast was prepared from fresh brewery yeast, pressed to half a ton per square inch and dried for 18–20 hours at 37°.

2 g. dried yeast + 10 ml. H_2O + 1–5 g. sugar were placed in a flask, immersed in a thermostat and connected to a nitrometer. Three or more fermentations were carried out at the same time with different sugars or different concentrations of the same sugar. The evolution of carbon dioxide was measured at intervals of 5 minutes and, when the rate had fallen to an approximately constant level, 2 ml. of a phosphate-sugar solution (0·5 *M* Na_2HPO_4 + *M* sugar, saturated with CO_2) were added. Further additions of this solution were made when the evolution of carbon dioxide had again fallen to the basal rate, the maximum number of additions being 6, each addition representing 1 millimol phosphate and 2 millimols sugar. At the end of the fermentation 3 ml. 25 % trichloroacetic acid were added, together with sufficient water to bring the total volume to 25 ml., and the solution was filtered.

Estimation of phosphates. Inorganic and total phosphates were estimated in the protein-free filtrate by the modified Briggs's method. Monophosphoric and diphosphoric esters were estimated in the following way. To 2 ml. of the filtrate, 1 ml. of a 50 % solution of barium acetate was added, and a solution of baryta was run in until the solution turned phenolphthalein faintly pink; 1 ml. absolute alcohol was added and the volume made up with water to 10 ml. The solution was heated in a water-bath at 70° for 5 minutes and filtered; it was then kept at 0° for 1 hour and again filtered if any further precipitate had formed. The total P in this filtrate represented the monophosphate fraction, the diphosphate being obtained by difference.

Separation of the monophosphate fraction. The main bulk of the trichloroacetic acid filtrate was treated with barium acetate, in amount equal to ten times the total P present, and then with baryta until the p_H was brought to 8·0. The precipitate (crude hexosediphosphate + inorganic phosphate) was removed by filtration and the filtrate treated with a solution of basic lead acetate in slight excess. The precipitated lead salt was separated by centrifuging, washed twice with water, decomposed with the minimum excess of sulphuric acid and again centrifuged to remove the lead sulphate, this being also washed several times with water. The solution was brought to p_H 8·0 with baryta, on which a small

amount of sparingly soluble barium salt was usually precipitated and this was removed by filtration (intermediate fraction). The filtrate was poured into three times its volume of alcohol and the precipitate (crude hexosemonophosphate) was filtered off, washed with alcohol and dried in an evacuated desiccator. After a further purification by solution in ten parts of water and re-precipitation with alcohol these barium salts were analysed by the methods described in previous papers.

Phosphoric esters produced from mannose, glucose and fructose under various conditions.

The effect of varying the initial concentration of sugar on the proportion of mono- and di-phosphoric esters produced during fermentation was investigated in a series of six experiments, the sugars used being mannose and glucose. In a further series of experiments the nature of the monophosphoric esters produced from mannose, fructose and glucose at 25° and 38° was examined more closely. Details of these experiments with the percentages of monophosphates produced are set out in Table I, while the analyses of the barium monophosphate fractions are given in Table II.

Two different preparations of dried yeast were used; the first, Y 9, was 3½ months old at the date of the first experiment, while the second, Y 10, was prepared shortly before use in Exp. 10. The duration of the preliminary period of fermentation, before the first addition of phosphate, ranged from about 60 minutes at 38° to 4 hours at 17°; the duration of the experimental period, from the first addition of phosphate to the addition of trichloroacetic acid, varied from 50 to 615 minutes according to the age of the yeast and the temperature of fermentation, as shown in Table I. The rates of evolution of carbon dioxide shown in the next two columns are (*a*) the basal rate at the end of the preliminary period, (*b*) the maximum value observed for a 5-minute interval between the first and second additions of phosphate. Owing to the rapid change in the rate these observed maxima have a limited significance but serve to show the marked falling off in the mannose-fermenting power of the yeast Y 9 which occurred during the course of the experiments. In Exp. 2, 6 millimols of phosphate were almost completely esterified in 180 minutes, the maximum rate of gas evolution being 13·0 ml. per 5 minutes. In Exp. 9, 5 millimols of phosphate were added during a period of 605 minutes and only 82% of this phosphate was esterified, the maximum rate of gas evolution being 5·2 ml. per 5 minutes. No such marked falling off was observed in the fermentative power of this yeast towards glucose, for in Exp. 9, 5 millimols of phosphate became esterified to the extent of 96% in 185 minutes, the maximum rate being 13·5 ml. per 5 minutes.

The total phosphate in the trichloroacetic acid filtrate included an amount, equivalent to about 40 mg. P, derived from the yeast; part of this was originally present as inorganic phosphate, becoming esterified during the preliminary fermentation; the remainder consisted of organic phosphates not necessarily identical with the esters formed during the fermentation.

In the fermentation of glucose at 34° (Exp. 1) the proportion of monophosphate was increased on raising the initial concentration of sugar, and reduced on increasing the total amount of added phosphate. On the other hand, in the fermentation of mannose at 38° (Exps. 2–4) the proportion of monophosphate was highest when the amount of sugar initially added was relatively small, 0·5–1 g., and was markedly reduced when this amount was increased to 3 or 5 g. Much smaller amounts of monophosphate were produced from mannose at 17° (Exp. 5), but in this case the proportion rose slightly with increasing concentration of sugar.

Table I. *Rates of fermentation and amounts of mono- and di-phosphoric esters produced from mannose, fructose and glucose under various conditions.*

Exps. 1–9: dried yeast, Y9, prepared 2. x. 31. Exps. 10–14: dried yeast, Y10, prepared 30. iii. 32.

				Additions				Rate of evolution of CO_2 ml. per 5 min.			
Exp.	Date	Temp. 0° C.	Sugar	Initial sugar g.	Total sugar millimols	Total phosphate millimols	Duration of experimental period min.	Basal	Maximum	Ester-P as % of total P	Monophosphate-P as % of total ester-P
1	21. i. 32	34	Glucose	1	10	2	65	3·0	15·5	98	21
,,	,,	,,	,,	3	21	2	55	,,	10·2	99	28
,,	,,	,,	,,	5	32	2	50	,,	9·2	98	34
,,	,,	,,	,,	3	25	4	105	,,	10·4	97	20
,,	,,	,,	,,	3	29	6	155	,,	11·3	95	19
2	26. i. 32	38	Mannose	1	10	2	60	3·0	11·8	99	66
,,	,,	,,	,,	3	21	2	60	,,	13·9	98	50
,,	,,	,,	,,	5	32	2	60	,,	10·0	97	42
,,	,,	,,	,,	3	25	4	120	,,	12·5	97	42
,,	,,	,,	,,	3	29	6	180	,,	13·0	95	38
3	2. ii. 32	38	Mannose	1	10	2	60	3·0	11·0	97	68
,,	,,	,,	,,	3	21	2	50	,,	16·5	97	44
,,	,,	,,	,,	5	32	2	60	,,	11·6	97	48
,,	,,	,,	,,	3	25	4	120	,,	13·8	96	44
,,	,,	,,	,,	3	29	6	180	,,	14·9	94	36
4	5. ii. 32	38	Mannose	0·5	7	2	85	2·5	8·1	98	60
,,	,,	,,	,,	1	10	2	85	,,	8·1	97	64
,,	,,	,,	,,	2	15	2	70	,,	15·5	97	50
,,	,,	,,	,,	3	25	2	65	,,	13·0	97	48
5	9. ii. 32	17	Mannose	0·5	7	2	210	0·7	2·9	99	27
,,	,,	,,	,,	1	10	2	210	,,	2·8	99	28
,,	,,	,,	,,	2	15	2	225	,,	2·2	99	30
,,	,,	,,	,,	3	25	2	210	,,	1·9	98	33
6	12. ii. 32	38	Mannose	1	10	2	65	2·5	10·1	99	58
,,	,,	,,	,,	3	21	2	55	,,	12·7	98	51
,,	,,	,,	,,	1	18	6	190	,,	9·5	96	74
7	1. iii. 32	38	Mannose	1	18	6	305	2·5	7·7	96	68
,,	,,	,,	,,	1	18	6	305	,,	6·6	98	79
,,	,,	,,	,,	1	18	6	305	,,	5·8	98	80
8	4. iii. 32	38	Mannose	1	16	5	615	2·0	3·7	75	51
,,	,,	,,	,,	1	16	5	615	,,	5·1	73	50
,,	,,	,,	,,	1	16	5	615	,,	4·0	76	54
9	9. iii. 32	38	Mannose	1	16	5	605	2·0	5·2	82	58
,,	,,	,,	Fructose	1	18	6	185	,,	13·2	96	29
,,	,,	,,	Glucose	1	18	6	185	,,	13·5	96	22
10	8. iv. 32	38	Mannose	1	18	6	180	3·0	15·5	97	65
,,	,,	,,	Fructose	1	18	6	170	,,	27·0	96	27
,,	,,	,,	Glucose	1	18	6	180	,,	19·0	96	21
11	20. iv. 32	38	Mannose	1	18	6	175	3·0	14·2	97	62
,,	,,	,,	,,	1	18	6	175	,,	14·7	97	66
,,	,,	,,	,,	1	18	6	175	,,	15·6	97	60
12	28. iv. 32	38	Mannose	3	29	6	160	3·0	15·2	96	51
,,	,,	,,	Fructose	3	29	6	160	,,	24·4	96	36
,,	,,	,,	Glucose	3	29	6	160	,,	19·5	96	21
13	9. v. 32	25	Mannose	3	29	6	350	2·0	13·1	97	37
,,	,,	,,	Fructose	3	29	6	350	,,	14·8	97	25
,,	,,	,,	Glucose	3	29	6	350	,,	13·4	97	18
14	14. v. 32	25	Mannose	3	29	6	350	2·0	12·8	95	36
,,	,,	,,	Fructose	3	29	6	350	,,	14·9	95	22
,,	,,	,,	Glucose	3	29	6	350	,,	13·3	95	18

Both at 38° and at 25° mannose yielded a much higher proportion of monophosphate than either fructose or glucose; and this result is in agreement with the findings of I. Neuberg and Ostendorf [1930] for fermentations at 37° with fresh yeast in presence of toluene. The highest value recorded in Table I is 80 %,

but values approaching 100 % have been obtained in this laboratory. With all three sugars the proportion of monophosphate was higher when the fermentation was carried out at 38° than at 25°. The highest yields were obtained from mannose at 38° with a relatively low concentration of sugar (initially 1 g. to 10 ml.); and under these conditions the difference between mannose and the other sugars was most pronounced. Fructose gave a little more monophosphate than glucose both at 38° and at 25°.

Nature of the monophosphate fractions. Analyses of the monophosphate fractions (crude soluble barium salts) revealed considerable variations in their composition (Table II). Those obtained from the fermentations of fructose and

Table II. *Analyses of monophosphate fractions (barium salts) isolated from Exps. 7–14.*

				Analysis of barium salt				
						Reducing power as glucose %		
Exp.	Temp. °C.	Sugar	Monophosphate-P as % of total ester-P	P %	Fructose (Selivanoff) %	H and J*	Iodine	$[\alpha]_{5461}$
4 and 6	38	Mannose	58	7·53	3·5	30·5	24·9	+ 4·3°
7	,,	Mannose	76	7·42	1·2	33·0	27·5	+ 3·0
8	,,	Mannose	52	6·70	2·1	28·7	22·8	+ 8·4
9	,,	Mannose	58	7·37	3·2	29·1	26·3	+ 8·4
	,,	Fructose	29	7·12	5·3	24·7	23·6	+12·2
	,,	Glucose	22	7·21	6·0	23·1	20·9	+12·4
10	,,	Mannose	65	7·70	1·7	30·2	23·7	+ 3·9
	,,	Fructose	27	7·79	8·0	23·0	17·2	+ 9·2
	,,	Glucose	21	7·67	6·8	20·8	14·8	+ 6·8
11	,,	Mannose	63	7·69	1·4	30·0	27·0	+ 3·0
12	,,	Mannose	51	7·65	2·3	30·6	24·4	+ 4·7
	,,	Fructose	36	7·57	7·8	27·7	19·2	+10·8
	,,	Glucose	21	7·36	9·5	22·7	16·7	+13·8
13	25	Mannose	37	7·38	4·3	30·4	20·3	+15·0
	,,	Fructose	25	7·14	7·1	22·8	16·7	+18·9
	,,	Glucose	18	7·11	6·7	21·6	15·2	+26·2
14	,,	Mannose	36	7·00	4·5	25·0	20·4	+16·8
	,,	Fructose	22	6·95	6·9	21·7	15·3	+23·7
	,,	Glucose	18	6·93	6·3	18·1	15·1	+26·5
	Glucosemonophosphate			7·86	0·5	35·5	45·7	+20·6
	Fructosemonophosphate			7·80	22·0	36·2	3·0	+ 0·7
	Mannosemonophosphate			7·87	0·3	36·0	28·5	+ 3·6

$C_6H_{11}O_5PO_4Ba$ requires P, 7·85 %; $C_6H_{12}O_6$, 45·6 %.

* Determined with addition of 0·5 ml. of 0·5*N* NaOH.

glucose were of the mixed type, containing 25–43 % of ketose esters, judged by the Selivanoff values, and a rather high proportion of the unknown ester of low reducing power. The high specific rotations of the products from fructose and glucose at 25° (Exps. 13, 14) pointed to the presence of trehalosemonophosphate, though in lower proportions than had been given by other samples of dried yeast [Robison and Morgan, 1928]. Very little, if any, trehalosemonophosphate was formed in the fermentations at 38°.

The monophosphate fractions obtained from mannose at 38° differed notably from those just considered and in their properties approximated more closely to mannosemonophosphate. The triple mannose fermentations of Exp. 7 yielded

monophosphate to the extent of 76 % of the total esterified phosphorus; and the analytical data suggested that over 90 % of this fraction consisted of the mannose ester. Very similar products, though of slightly lower reducing power, were obtained in Exps. 10 and 11.

Apart from the specific rotations, the low Selivanoff values are especially to be noted, as showing that ketose esters were present in very small amounts, probably not exceeding 5 %. Other mannose fermentations at 38° (Exps. 4–6, 8, 9, 12), in which the yields of monophosphate were a little lower, gave products containing higher proportions of fructose and glucose esters, though still consisting for the most part of mannosemonophosphate.

Fermentation of mannose at 25° yielded monophosphate fractions of intermediate character, whose composition was more difficult to judge from the analytical data. The Selivanoff values indicated about 20 % of ketose esters; the specific rotations were similar to those of the usual mixed ester, but would also be consistent with the presence of much mannosemonophosphate and a little trehalosemonophosphate. It is shown later that the latter explanation is more probably correct.

Separation of mannosephosphate from the products of mannose fermentations at 38°.

(*a*) *By the brucine salt.* The products from Exps. 7 and 11 which had apparently yielded the highest proportions of mannose ester were converted into the brucine salt which was recrystallised five times from 80 % methyl alcohol. The main fraction, after re-conversion into barium salt, gave analytical values in agreement with those previously obtained for mannosephosphate but the Selivanoff value had not been lowered; there was indeed evidence that unduly prolonged contact with the solvent had led to some increase of ketose esters.

(*b*) *By the phenylhydrazone.* Formation of the phenylhydrazone provided a simpler and more satisfactory method of isolation of the mannose ester and one that could be directly applied to mixtures containing this ester in relatively small proportions. It was found possible to remove both phenylhydrazine groups from this derivative by treatment with benzaldehyde at room temperature, the benzaldehydehydrazone and excess of benzaldehyde being subsequently removed by extraction with ether. In this way the recovery of the free mannose ester was effected under conditions involving no likelihood of intramolecular change.

The combined soluble barium salts from the mannose fermentations at 38° of Exps. 4, 6, 8, 9, 10, 12, were converted into the free esters by treatment with the minimum excess of sulphuric acid, the barium sulphate being removed by centrifuging and washed four times with acidified water. The ester solution was treated with pure phenylhydrazine (4 mols.) and the equivalent amount of acetic acid and was then left in an evacuated desiccator for some hours. The phenylhydrazine salt of phosphomannosephenylhydrazone which separated out was filtered off and washed with cold methyl alcohol; the yield corresponded with 60 % of the total P present in the barium salt. The hydrazone was recrystallised from 50 % ethyl alcohol, the operation being conducted as rapidly as possible. This recrystallisation, which involved considerable reduction in the yield, was later found to be unnecessary as a stage in the isolation of the pure ester.

The hydrazone was decomposed by treating it with about ten times its weight of benzaldehyde, in which it dissolved almost completely. After leaving for an hour at room temperature, an equal volume of water and twice the volume of ether were added, and the mixture was gently shaken in a separating

funnel. The aqueous layer was removed and shaken with a further quantity of benzaldehyde during one hour at room temperature; it was then extracted with ether until the extract was colourless and gave a negligible residue on evaporation. The aqueous solution, which now contained the free mannosemonophosphoric ester, was brought to p_H 8·0 by addition of baryta and poured into three times its volume of alcohol. The precipitated barium salt was purified by solution in 10 % alcohol and reprecipitation in 70 % alcohol.

Analyses of the salts obtained in this way agreed well with those of mannosemonophosphate but the Selivanoff value was still between 1 and 2 %.

(*c*) *By the crystalline barium salt.* During purification of the barium salt obtained from the phenylhydrazone it was observed to separate from dilute aqueous alcohol in a crystalline and sparingly soluble form. As in the case of barium trehalosemonophosphate, when once the crystalline form had been obtained it separated readily from any solution in which the ester was present in moderate concentration. Thus 1·2 g. of the amorphous barium salt, which had been several times dissolved in 10 parts of water, suddenly separated out as a mass of crystals which thereafter required 150–200 ml. of water for complete solution. From this solution, on gradual addition of alcohol up to 20 % concentration, the barium salt separated slowly at room temperature in clusters of fine needles; they were recrystallised in similar manner from 20 % alcohol and were analysed.

(The crystalline salt lost 8·4 % at 110° in the Pregl drier. Required for $2H_2O$, 8·4 %. Found for the dry salt: C, 18·02, 17·90; H, 3·2, 2·90; Ba, 34·5, 34·7; P, 7·87, 7·85 %. $C_6H_{11}O_5PO_4Ba$ requires C, 18·20; H, 2·81; Ba, 34·75; P, 7·85 %.) C and H were determined by the Pregl method, the finely divided substance being mixed with potassium dichromate and copper oxide; as with other hexosephosphates, complete combustion was very difficult to effect. Other analytical data for this salt are shown at the foot of Table II. The values for specific rotation and reducing power do not differ significantly from those previously recorded [Robison, 1932]; but the Selivanoff value has been reduced from 1 to 0·3 % which is no more than that given by pure mannose. Recrystallisation of the barium salt had therefore removed the last traces of ketose esters. The H. and J. reducing power, determined with addition of NaOH, is the same as that of glucose- and fructose-6-phosphates and is 80 % of that of the equivalent amount of mannose. The iodimetric reducing power is only about 60 % of that theoretically required for an aldosemonophosphate. As previously stated, it can be raised to about 70 % by increasing the concentration of potassium iodide in the iodine solution. Further tests showed that, under the normal conditions of this iodimetric method, mannose itself gives somewhat low values, which are slightly raised if the concentration of potassium iodide is increased. By extending the period of oxidation, still higher values were obtained; but the oxidation of the ester proceeded more slowly and was less complete than that of the free sugar. Thus in two hours the oxidation of mannosemonophosphate corresponded with 87 % of the theoretical value, while that of mannose reached 100°. Under similar conditions fructose was oxidised only to the extent of 2 %. The typical results, shown in Table III, suggest that the ester exists in solution in two forms, only one of which is rapidly oxidised by iodine.

Although the separation of mannose ester in these operations was not quantitative, the conclusions deduced from the analyses of the crude fractions were definitely confirmed—namely that these fractions consisted to a very large extent of mannosemonophosphate, which therefore constitutes the main phosphorylated product of the fermentation of mannose by dried yeast at 38°.

Table III. *Iodimetric reducing power of mannosemonophosphate.*

3 ml. 0·02 *N* I; 0·2 ml. 5 % Na_2CO_3; $t=22°$; normal time, 30 min.

Substance	Weight of substance mg.	Time min.	Extra KI mg.	Reducing power as aldose: % of substance	Reducing power as aldose: % of equivalent hexose
Barium mannose-phosphate	3	30	—	28·5	62
	〃	15	30	26·9	59
	〃	30	〃	32	70
	〃	60	〃	36·4	80
	〃	120	〃	40·6	87
Mannose	1	30	—	92	92
	〃	15	30	89	89
	〃	30	〃	96	96
	〃	60	〃	97	97
	〃	120	〃	100	100
Fructose	5	30	—	0·5	0·5
	〃	30	30	0·5	0·5
	〃	60	〃	1·2	1·2
	〃	120	〃	2·0	2·0

Separation of mannosemonophosphate from the products of mannose fermentations at 25° and of glucose and fructose fermentations at 25° and 38°.

The analyses of the monophosphate fractions obtained from mannose fermentations at 25° and from the fermentations of glucose and fructose at 25° and 38° had given no clear evidence as to the presence of mannosemonophosphate in these mixtures of aldose and ketose esters. The fractions were therefore dissolved separately in 10 parts of 10 % alcohol and the filtered solutions were seeded with a minute crystal of barium mannosephosphate and left for some days at 0°. Separation of crystalline barium salt was observed to take place from the products obtained from mannose at 25° (Exps. 13 and 14) but not from any of the products obtained from glucose or fructose. Analysis of the crystalline product from Exp. 13, mannose, showed that this was barium mannose-monophosphate; the residual salt, precipitated from the mother-liquors by alcohol, had an increased specific rotation ($[\alpha]_{5461}+23°$) and most probably contained trehalosemonophosphate. The weight of crystalline mannosephosphate recovered amounted to 18 % of the fraction, but almost certainly this did not represent the whole of the mannose ester present. The phenylhydrazone was also prepared, and after recrystallisation from 75 % alcohol, melted at 144–144·5°, both when heated alone and when mixed with pure mannosephosphate hydrazone.

Formation of the hydrazone was also used to demonstrate the presence of mannosemonophosphate in the monophosphate fractions from glucose and fructose fermentations at 38° (Exps. 9, 10 and 12) and at 25° (Exp. 13). Although these products failed to yield the crystalline barium mannosephosphate, all gave small amounts of sparingly soluble hydrazones which, after recrystallisation, melted at 144–145° and did not lower the melting-point of pure mannosephosphatehydrazone.

It appears, therefore, that mannosemonophosphoric ester is a normal product of the fermentation of glucose, fructose and mannose by dried yeast both at 25° and at 38°, but that it occurs in considerably larger amounts in the products obtained from mannose than in those from glucose and fructose.

Intermediate fractions obtained from the fermentation products.

Analysis of the intermediate fractions obtained in the separation of the barium salts gave results consistent in all cases with the view that these fractions might be composed chiefly of hexosediphosphate, mixed with a little hexosemonophosphate, inorganic phosphate and, possibly, also with phosphoglycerate. It was noted, however, that such intermediate fractions were not obtained in appreciable amounts from mannose fermentations at 38°, which suggests that some unknown products of intermediate solubility may be formed from glucose and fructose and also from mannose at 25° but not from mannose at 38°.

Phenylhydrazine salt of mannosemonophosphatephenylhydrazone.

A specimen of the phenylhydrazone prepared from pure mannosemonophosphate and recrystallised from 75 % alcohol was very pale yellow in colour, slightly soluble in water and methyl alcohol, less soluble in ethyl alcohol, ether, chloroform and acetone; M.P. 144–144·5°. (Found for the dry substance: P, 6·80; N (micro-Dumas), 12·2 %. $C_{18}H_{27}O_8N_4P$ requires P, 6·78; N, 12·23 %.)

Discussion.

If the simplified methods for the isolation of pure mannosemonophosphate which were evolved during the course of this investigation could have been applied throughout the whole series of fermentation experiments, the quantitative value of the results would have been increased. Nevertheless these results show that mannosemonophosphate is normally formed in the fermentation of glucose, fructose and mannose by dried yeast in presence of phosphate at 25° and at 38° but that it occurs in considerably larger amounts in the products from mannose than in those from glucose and fructose. This difference is apparent in fermentations at 25° but is very much exaggerated when the temperature of fermentation is raised to 38°; in such cases mannosemonophosphate may form almost the whole of the monophosphate fraction and the chief part of the total phosphorylated products.

Such conclusions make it necessary to reconsider the scheme put forward in a previous paper [Robison, 1932] according to which the first stage in the fermentation of glucose, fructose and mannose is the formation of the enol compound common to all three sugars; the phosphoric acid group then enters position 1 of this enol compound. Clearly this view is inconsistent with the production of mannosephosphate to such preponderating extent in the fermentation of mannose. Rather it would seem that the phosphoric group may enter position 6 in the molecule of unchanged hexose and that enolisation of the hexosemonophosphate then takes place. Enolisation of mannose-6-phosphate may occur less readily than that of the corresponding esters of glucose and fructose at 25° and may be inhibited almost completely at 38°. Since fructosediphosphate could obviously not be formed unless enolisation first took place, this explanation would also account for the very small proportion of diphosphate in the phosphorylated products from mannose fermentations at 38°.

Before this argument can be continued, however, it is necessary to know whether the formation of mannosemonophosphate in large amount occurs as a normal stage in the fermentation cycle or as a result of a side reaction, a simple phosphorylation, unconnected with the carbohydrate breakdown. This can be investigated by determining the ratio of carbon dioxide evolved to phosphate esterified under conditions involving the formation of mannosemono-

phosphate in large as well as in small amounts. In the present series of experiments, although carbon dioxide evolution was measured, the corrections necessary for the accurate determination of this ratio could not be applied. A further series of experiments has since been carried out in order to study this aspect of the question; and these will be discussed in a subsequent communication (Patwardhan and Robison, unpublished results).

The preparation of the phenylhydrazine salt of the phenylhydrazone of mannosemonophosphate and its decomposition by benzaldehyde have supplied a satisfactory method of separating this ester from other monophosphates; while the sparingly soluble crystalline barium salt has furnished a simple means of obtaining the ester in pure condition. By a combination of these two processes the detection and approximate estimation of mannosemonophosphate in mixed phosphorylated products becomes a relatively simple operation which may be usefully applied in future investigations.

Summary.

1. The monophosphoric esters formed in the fermentation of mannose, glucose and fructose by dried yeast at various temperatures have been studied.

2. A method for the isolation of mannosemonophosphoric ester by formation of its phenylhydrazone and decomposition of the latter with benzaldehyde at room temperature is described.

3. The barium salt of mannosemonophosphate has been obtained in a sparingly soluble crystalline form which has furnished a simple means of obtaining the ester in pure condition.

4. The properties of this pure mannosemonophosphoric ester are described; they agree in nearly all respects with those recorded in the previous communication.

5. Mannosemonophosphate is formed in the fermentation of glucose, fructose and mannose by dried yeast in presence of phosphate at 25° and at 38°.

6. It is formed in considerably larger amounts from mannose than from glucose or fructose. This difference is apparent in fermentations at 25° but is greatly exaggerated when the fermentation is carried out at 38°.

Mannosemonophosphate may thus constitute almost the whole of the monophosphate fraction and the chief part of the phosphorylated products formed in the fermentation of mannose at 38°.

7. The bearing of these facts on previous views regarding the first stages in the fermentation of sugars is discussed.

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CCXLIV. MANNOSEMONOPHOSPHATE.
III. PHOSPHOMANNONIC ACID AND ITS LACTONES.

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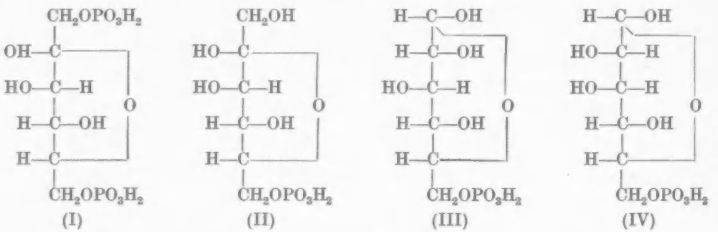
On the basis of the investigations of Young [1909; 1911], Morgan [1927; 1929], Morgan and Robison [1928] and Levene and Raymond [1928] it appears probable that the hexosediphosphoric ester of Harden and Young has the constitution (I).



The occurrence of the Robison ester (hexosemonophosphates) side by side with the Harden and Young ester in the products of fermentation was an indication that some structural relationship existed between these compounds; and the method of preparation of the Neuberg ester (II) by partial hydrolysis of hexosediphosphate was a proof of its genetic relation with the Harden and Young ester.

Robison and King [1931] succeeded by repeated crystallisation of the brucine salts of the mixed monophosphates of fermentation in isolating pure glucose-monophosphate for which they proposed the structure (III).

They also isolated from the same source a fructosemonophosphate identical with the Neuberg ester.

Robison [1932] isolated another ester from the products of fermentation of glucose and fructose with yeast preparations, which he showed to be a derivative of mannose. It gave the same osazone as the three esters previously mentioned and very probably in this case too the phosphate radical occupies position 6 in the hexose molecule.

The experimental facts on which these formulae are based are summarised later in the paper, and additional evidence has been obtained which forms the subject of the present communication.

Neuberg, I. S. and Ostendorf [1930] observed that in the fermentation of mannose with fresh yeast and toluene at 37° large amounts of monophosphates

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were formed. Their crude monophosphate fraction showed a high specific rotation which was possibly due to the presence of trehalosemonophosphate. After the isolation of pure mannosemonophosphate by Robison [1932] from the products of fermentation of glucose and fructose by dried yeast, Jephcott and Robison [1933; 1934] were able to show that at 37° the fermentation of mannose with dried yeast yields a large proportion of monophosphate which is mostly mannosemonophosphate. From these fractions they isolated the mannosemonophosphate in pure condition by the formation of the phenylhydrazone and its subsequent decomposition by benzaldehyde followed by the crystallisation of the barium salt of the ester. Their conclusions have been confirmed in the present investigation and large amounts of mannosemonophosphate have been prepared by a method based on these observations.

Experimental.

Experiments which will be described in a subsequent paper have shown that mannose when fermented with yeast juice or zymin at 37° does not yield a monophosphate fraction rich in mannosemonophosphate. Dried brewer's yeast is the only type of yeast preparation that is known to give rise to such large amounts of the mannose ester. When mannose was fermented with dried baker's yeast at 37° the yield of the monophosphate fraction was low and the nature of this fraction differed considerably from that of the product obtained from the fermentation of mannose with dried brewer's yeast under similar conditions. Table I shows the difference between the crude monophosphate fractions obtained by fermenting mannose with different yeast preparations at 37°.

Table I. *Fermentation of mannose with different yeast preparations at 37°. Analyses of the monophosphate fractions (barium salts).*

Yeast preparation	Monophosphate-P as % total ester-P	P %	Selivanoff fructose %	Reducing power as hexose % H. and J.	Reducing power as hexose % Iodine	$[\alpha]_{5461}$
Dried baker's yeast	23·2	6·30	6·3	16·5	9·1	+29·4°
"	29·5	6·51	6·1	17·5	9·7	+39·6
Dried brewer's yeast	85·5	7·25	0·8	29·0	19·4	+ 3·9
Yeast juice	63·8	6·17	4·7	24·8	21·0	+19·7
Zymin	39·8	6·65	5·0	21·7	19·4	+14·2

Preparation of mannosemonophosphate.

Mannosemonophosphate was prepared by fermenting mannose at 37° with dried English brewery yeast, as described by Jephcott and Robison [1934]. 50 g. dried yeast were mixed with 25 g. mannose and 250 ml. water; after the preliminary period of fermentation a 0·5 *M* solution of Na_2HPO_4 containing 18 % mannose was added at such a rate (10 ml. every 5 minutes) that a nearly constant high rate of fermentation was maintained until a total of 300 ml. solution had been introduced: trichloroacetic acid was then added to give a 4 % concentration. The monophosphate fraction represented 74 % of the esterified P; yield of air-dry crude barium hexosemonophosphate, 43·3 g. The analysis of this salt (Table II) indicated that it probably contained nearly 90 % of mannosephosphate.

Purification through the phenylhydrazone. 12 g. of the crude barium mannosemonophosphate were converted into the free ester and from this the phenylhydrazine salt of phosphomannosephenylhydrazone was prepared. It began to

separate within a few minutes and, after 3 hours at room temperature, was filtered and washed with ice-cold 10 % methyl alcohol in ether and dried *in vacuo*; yield, 9·4 g., nearly 80 % of the amount calculated on the P content[1]. It was decomposed by shaking for 3 hours with 25 g. benzaldehyde and 30 ml. water, extracting with ether, again shaking with benzaldehyde and re-extracting with ether. The free mannosemonophosphoric acid in the aqueous layer was converted into barium salt and precipitated with alcohol. The dry salt was redissolved in 10 parts of water and one of alcohol and filtered; crystallisation of the barium salt started at once but was allowed to proceed overnight at 0°. It was further purified by three or four crystallisations from 20–30 % aqueous alcohol.

The mother-liquors obtained after filtering off the sparingly soluble phenylhydrazone were also decomposed with a large excess of benzaldehyde and converted into the barium salts.

Purification by direct crystallisation from the crude monophosphate fractions. Barium mannosemonophosphate was also found to crystallise directly from aqueous solutions of the crude monophosphate fractions when present in concentrations higher than about 40 %. Crystallisation was induced by seeding a 10 % aqueous solution of the crude monophosphate with a few crystals of barium mannosemonophosphate. The salt was recrystallised from aqueous alcohol and, as the following analyses show, was of the same degree of purity as the recrystallised barium salt obtained by the decomposition of the phenylhydrazone. For purposes of comparison the analyses of the residues recovered from the phenylhydrazone mother-liquors and from the mother-liquors after direct crystallisation of the barium mannosemonophosphate fractions are also given in Table II.

Table II. *Analyses of the monophosphate fractions during purification.*

Ester	P %	Selivanoff fructose %	Reducing power as glucose %		$[\alpha]_{5461}$
			H. and J.	Iodine	
(A) Crude monophosphate; starting material	7·18	1·5	33·9	23·4	+ 2·6°
(B) Crystalline Ba salt by direct crystallisation from (A)	7·80	0·3	35·8	26·6	Free acid +16 (c = 1·68)
(C) Crystalline Ba salt from the hydrazone prepared from (A)	7·84	0·3	36·3	24·5	
(D) Ba salt from the mother-liquor of (B)	6·88	4·3	25·8	15·9	+ 2·2
(E) Ba salt from the mother-liquor of phenylhydrazone	6·62	5·4	19·6	19·0	+12·5

Monophosphomannonic acid. The oxidation of mannosemonophosphate.

5 g. of the crystalline barium mannosemonophosphate were dissolved in water, and bromine and baryta were added in three portions at intervals of 10 minutes so that both reagents were finally present in 33 % excess [Macleod and Robison, 1933]. The mixture was kept at room temperature, and 40 minutes after the first addition of bromine and baryta it was acidified with dilute HCl. Excess of bromine was driven off by aeration and the acid barium salt of

[1] Starting with pure barium mannosemonophosphate, the yield of hydrazone obtained by the same procedure varied from 70 to 80 % of the calculated weight.

phosphomannonic acid was precipitated at p_H 4·0 by 3 volumes of 96 % alcohol. The precipitate was filtered, washed with absolute alcohol and dried *in vacuo* over sulphuric acid. The dry acid salt was redissolved in a large volume of water and the solution was brought to p_H 8·0 with baryta and poured into twice its volume of alcohol; the neutral salt separated out as an amorphous mass which was filtered off and dried. It was further purified by redissolving in water and reprecipitating with alcohol. (Found for the dry neutral salt: P, 6·48; Ba, 43·24 %; $(C_6H_{10}O_{10}PBa)_2Ba$ requires P, 6·47; Ba, 43·01 %.)

Transformation of phosphomannonic acid into its lactones.

Phosphomannonic acid in aqueous solution undergoes a spontaneous transformation into the lactones, a change which can be followed by polarimetric observations. This property is common to all hydroxycarboxylic acids in which the hydroxyl is in the γ- or δ-position with respect to the carboxyl. The following experiments illustrate such a change; the isolation of the lactones is described later.

(*a*) *In aqueous solution.* 88·6 mg. of the barium phosphomannonate were decomposed with the calculated amount of *N* sulphuric acid, the volume was made up to 5 ml. and the $BaSO_4$ separated by centrifuging. The clear centrifugate was kept at 24° and polarimetric readings were taken at intervals. The results are given in Table III and Fig. 1, curve *C*, the rotations being calculated for the free acid.

Table III. *Lactone formation in aqueous solution at 24°.*

Time	(α)	$[\alpha]_{5461}$
20 min.	−0·005°	− 1·0°
1 hour	+0·026	+ 5·2
1½ hours	+0·030	+ 6·1
2½ „	+0·040	+ 8·2
20 „	+0·065	+13·3
2 days	+0·105	+21·4
4 „	+0·116	+23·7

(*b*) *In dilute HCl.* 0·1267 g. barium phosphomannonate was dissolved in water with 1 ml. of *N* HCl and the volume made to 10 ml. The excess of HCl over that required for the complete liberation of phosphomannonic acid was such that the final concentration of free HCl was 0·025 *M*. The solution was kept at 24°, and the polarimetric observations were made in a 1 dm. tube. The numbers in Table IV and curve *A*, Fig. 1, show the change in rotation due to lactone formation, the rotations being calculated for the free acid.

Table IV. *Lactone formation in 0·025* M *HCl at 24°.*

Time	(α)	$[\alpha]_{5461}$
0 hour	−0·010°	− 1·4°
1 „	+0·046	+ 6·6
4 hours	+0·075	+10·7
9 „	+0·100	+14·4
25 „	+0·125	+17·9
7 days	+0·204	+29·5

After 4 hours a portion of the liquid was heated in a sealed tube at 100° for 1 hour. The rotation rose to +34·6° and remained unchanged after further

heating (curve *B*, Fig. 1). Fig. 1 illustrates the course of lactone formation under different conditions; it appears from curve *A* that two reactions are proceeding simultaneously, one slow and the other rapid, a fact which indicates the formation of two lactones.

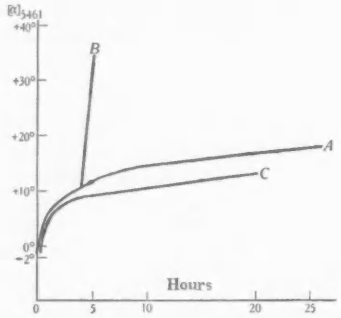

Fig. 1. Lactone formation from 6-phosphomannonic acid. *A*, in 0·025 *M* HCl at 24°; *B*, at 100°; *C*, in aqueous solution at 24°.

Preparation of phosphomannono-γ- and phosphomannono-δ-lactone.

It is known [Haworth, 1929] that heating a hexonic acid at 100° for a short time leads to the formation of γ-lactone, while heating at lower temperatures for prolonged periods favours the formation of δ-lactone. This observation has been made use of in the isolation of the phosphomannonolactones.

2 g. of the barium phosphomannonate were decomposed with the calculated amount of *N* H_2SO_4, and the free acid was separated from $BaSO_4$ by centrifuging and washing the residue repeatedly with water. The combined centrifugates were divided into two portions, one being heated in a sealed tube at 100° for one hour and then evaporated to dryness *in vacuo* over sulphuric acid, while the other portion was evaporated to dryness *in vacuo* at room temperature without previous heating. In both cases the lactones separated in star-shaped clusters of small needles after the evaporation of the solvent. Attempts to recrystallise them from alcoholic solutions failed; hence they were purified by washing with cold anhydrous acetone, redissolving in water, filtering and evaporating to dryness *in vacuo* over sulphuric acid and finally over phosphorus pentoxide. The properties and analyses of the pure compounds are given below.

Phosphomannono-γ-lactone: M.P. 125–125·5°; P, 12·10%; $[\alpha]_{5461}$ +54·1°.
Phosphomannono-δ-lactone: M.P. 127·5–128·5°; P, 12·07%; $[\alpha]_{5461}$ +60·6°.
$C_6H_{11}O_9P$ requires P, 12·01 %.

Hydrolysis of the phosphomannonolactones in aqueous solution.

34·7 mg. of the lactone were dissolved in 5 ml. water and the polarimetric readings taken in a dm. tube at 24° (Table V). The amount of free acid present in aqueous solution at any given moment is calculated from the expression [Drew *et al.*, 1927]

$$\frac{(R-R_l)}{100\,(R_a-R_l)},$$

where R is the specific rotation at any given time, R_a and R_l the specific rotations of the substance in form of the acid and the lactone respectively. The value of R_a is arrived at from the expression

$$r_a \cdot \frac{M_a}{M_l},$$

where r_a is the true specific rotation of the acid and M_a and M_l the molecular weights of the acid and the lactone respectively.

Table V. *Hydrolysis of lactones in aqueous solution at 24°.*

	γ-lactone		δ-lactone	
Time in days	$[\alpha]_{5461}$	Free acid %	$[\alpha]_{5461}$	Free acid %
0	+54·1°	0·0	+60·6°	0·0
1	+53·6	1·0	+58·2	3·8
2	+53·4	1·4	+55·2	8·3
4	+50·5	6·5	+50·9	15·0
6	+48·6	10·0	+47·0	20·9
8	—	—	+43·6	30·7
15	+43·1	19·8	—	—
19	—	—	+43·6	—
30	+43·1	—	—	—

The δ-lactone is more readily hydrolysed in aqueous solution than the γ-lactone; ultimately, however, an equilibrium would be attained between the two lactones and the free acid. It is true that the percentages of free acid calculated are different in the two cases, but the close agreement between the final values of the specific rotations suggests that equilibrium has been reached. Clearly the above formula by which these values for the free acid were calculated is not strictly applicable to this case since three variants instead of two are present in the solution.

Hydrolysis of monophosphomannonic acid by N *HCl at 100°.*

49·7 mg. of barium phosphomannonate together with 26·1 mg. anhydrous potassium sulphate were dissolved in 10 ml. *N* HCl. Barium sulphate was separated by centrifuging and the clear solution measured into small ampoules, 1·2 ml. in each. The ampoules were then sealed and immersed in a boiling water-bath. At definite intervals one ampoule was taken out, cooled in water and the inorganic phosphate content of the solution determined. Table VI shows the rates of dephosphorylation of the phosphomannonic acid and also of phosphogluconic acid under similar conditions. The latter was a pure sample kindly supplied by Prof. Robison [Robison and King, 1931]. The constant k was calculated according to the equation for unimolecular reactions

$$k = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2},$$

where x_1 and x_2 represent the amounts hydrolysed at the times t_1 and t_2 respectively, a being the total concentration of the unhydrolysed acid at the beginning of the hydrolysis.

The higher values of k for the first 8 hours are peculiar in that they seem to be common to both phosphomannonic and phosphogluconic acid. This suggests that the compounds undergoing hydrolysis during that period are different from those being hydrolysed in the later stages. It is possible that the free phosphohexonic acids are hydrolysed at faster rates than the corresponding

Table VI. *Hydrolysis of phosphomannonic and phosphogluconic acids in N HCl at 100°.*

Time in hours	Phosphomannonic acid		Phosphogluconic acid	
	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$
4	10·4	0·199	13·8	0·267
8	19·2	0·187	23·9	0·228
24	38·6	0·124	45·9	0·154
48	60·8	0·136	67·7	0·155
72	72·9	0·111	—	—
96	82·4	0·131	—	—

lactones which are formed by heating the acids in *N* HCl at 100°. Only when all the acid is converted into the lactone is a constant rate attained. That such is possibly the case is shown by the hydrolyses of phosphomannono-γ- and phosphomannono-δ-lactones which are set out in Table VII. In both cases the rates of hydrolysis of the phosphoric ester linkage were constant from the beginning.

Table VII. *Dephosphorylation of phosphomannonolactones in N HCl at 100°.*

Time in hours	γ-lactone		δ-lactone	
	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$
4	6·9	0·129	6·3	0·117
8	12·7	0·116	12·4	0·122
24	32·6	0·117	32·2	0·116

12·9 mg. of the lactone were dissolved in 5 ml. of *N* HCl and the hydrolysis investigated by the procedure already described.

Discussion.

Mannosemonophosphoric ester on mild oxidation yields phosphomannonic acid which in aqueous solution or in presence of dilute HCl is transformed into its lactones. By heating the free acid in aqueous solution at 100° for one hour a lactone has been isolated which is probably the γ-lactone; on the other hand slow evaporation of the aqueous solution at room temperature has led to the isolation of another derivative which is probably the δ-lactone. The properties of the two compounds are given below; the figures for the corresponding unsubstituted mannonolactones, quoted from Haworth and Nicholson [1926], are shown for comparison.

Lactone	M.P.	$[\alpha]_D$	$[\alpha]_{5461}$
Phosphomannono-γ-lactone	125–125·5°	—	+54·1°
Phosphomannono-δ-lactone	127·5–128·5	—	+60·6
γ-Mannonolactone	151	+ 51·8°	—
δ-Mannonolactone	156	+114	—

The transformation of phosphomannonic acid into its lactones and the rates of hydrolysis of these lactones in aqueous solution appear to be in general agreement with the known behaviour of the corresponding substituted and unsubstituted sugar acids and their lactones. Hence it is probable that the designations γ-lactone and δ-lactone for the two isolated phosphomannonolactones are correct, although further confirmation would be desirable.

Assuming then that these two lactones are the 1:4- and 1:5-lactones of phosphomannonic acids the further conclusion would follow that the positions 4 and 5 in the acid and therefore in the parent mannosemonophosphoric ester must be free, as otherwise only one lactone should have been formed. The evidence which supports the conclusion that the phosphate radical in mannosemonophosphate occupies position 6 can be summarised as follows.

(1) Hexosediphosphoric acid yields a phenylhydrazone containing two phosphate radicals, but during the formation of the osazone one molecule of phosphoric acid is split off [Young, 1911]. One of the two phosphoric acid groups must therefore occupy position 1 in the hexosediphosphate molecule.

(2) Hexosediphosphoric acid on methylation forms the α- and β-methylglycosides [Morgan, 1927], which on dephosphorylation with bone-phosphatase yield the methylglycosides identified as derivatives of fructfuranose [Morgan and Robison, 1928].

(3) Hexosediphosphoric acid on partial hydrolysis with dilute hydrochloric acid [Neuberg, C., 1918] yields a hexosemonophosphoric acid which is a derivative of fructose and which yields an osazone identical with the osazone from the hexosediphosphoric acid [Neuberg, C. and Reinfurth, 1924]. Fructosemonophosphate (Neuberg ester) therefore does not contain the phosphate radical which occupied position 1 in hexosediphosphoric acid.

(4) Glucosemonophosphate isolated by Robison and King [1931] from fermentation hexosemonophosphate yields on methylation α- and β-methylglycosides of the furanose and pyranose type [King *et al.*, 1931] which indicates that positions 4 and 5 in the glucosemonophosphate molecule are free.

(5) An ester, probably glucose-6-phosphate, has been synthesised by Levene and Raymond [1931] and appears to be identical with the glucosemonophosphate of Robison and King.

(6) Glucosemonophosphate and mannosemonophosphate yield the same osazone [Robison, 1932], which is also identical with that obtained from fructosemono- and fructosedi-phosphate. The phosphate radical therefore must occupy identical positions in all the three monophosphoric esters.

(7) An enzyme from yeast (kinase) converts any of these three monophosphoric esters in aqueous solution into equilibrium mixtures containing all the three esters [Lohmann, 1933; Patwardhan and Robison, unpublished experiments]. This points to the same conclusion as arrived at in the preceding paragraph.

(8) Mannosemonophosphate on mild oxidation yields phosphomannonic acid which forms two lactones whose properties correspond with those of the γ- and δ-lactones of the sugar acids.

The whole of this evidence put together points to the conclusion that the phosphoric acid group in mannosemonophosphate occupies position 6 in the mannose chain. This confirms the conclusions previously arrived at not only with regard to the constitution of mannosemonophosphate but also to that of the glucose and fructose esters isolated from the products of fermentation.

SUMMARY.

1. Large amounts of mannosemonophosphate have been prepared by the fermentation of mannose with dried brewer's yeast at 37° followed by crystallisation of the barium salt from the crude monophosphate fractions.

2. Mannosemonophosphate has been oxidised with bromine in presence of baryta to yield phosphomannonic acid which in aqueous solution passes into the lactone form.

3. Phosphomannono-γ- and phosphomannono-δ-lactone have been isolated and their rates of hydrolysis in aqueous solutions studied.

4. The rate of dephosphorylation of the phosphomannonic acid in *N* HCl at 100° falls after the first 8 hours and then attains a constant value which is equal to the rate of dephosphorylation of the corresponding lactones under comparable conditions. Hence it is suggested that the free acid is dephosphorylated by *N* HCl at 100° at a higher rate than its lactones.

5. The formation of these two lactones furnishes evidence that positions 4 and 5 in the mannose chain are free, and this taken in conjunction with previous findings points to the correctness of the conclusion that phosphate group occupies position 6 in mannosemonophosphate.

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CCXLV. EXPERIMENTS ON NUTRITION.
XIII. THE RELATIVE VALUES OF PROTEINS.

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As there are excellent reviews of the voluminous literature on the subject of the comparative or biological values of proteins in nutrition, it is not necessary to recapitulate. We may refer especially to McCollum and Simmonds, *The Newer Knowledge of Nutrition*, 3rd edition, 1927, in which book there is a very full bibliography, to Mitchell's "The nutritive value of proteins" in *Physiological Reviews*, 1924, and to the papers of Boas-Fixsen and Jackson in this *Journal*, 1932; 1934. Boas-Fixsen and Jackson describe clearly the three methods commonly used in these investigations. Information about the comparative values of the proteins in the common foodstuffs, such as milk, meat, fish, eggs, pulses, green leaves, used in conjunction with a cereal food, such as wheat, as in everyday life, is difficult to find, and the available data are conflicting as they have been secured by workers making different kinds of experiments. We have therefore during the last seven or eight years attempted to find out the comparative and relative values of various proteins in the same general diet. At the same time it was of interest to examine the values of the proteins of various cereals without the supplement of other proteins. The experiments have been made by the growth method on chickens instead of on rats, because there is little information on the growth of these birds and because they grow almost ten times as much as rats in the same time on a good combination of protein and cereal. The differences would therefore be expected to be greater and the results more definite. The results are not however so clear as we had hoped they would be, and only after long discussion do we offer them for publication in as short a description as we are able to write.

Experimental.

Chickens.

Our previous experiments with chickens were made with the Light Sussex variety. As it was inadvisable to change, this breed was again used. We hoped to be able to procure eggs from the same stock of parent birds for all the experiments, but the supplies were too intermittent and the hatching so irregular that this ideal had to be abandoned. We fortunately discovered a professional supplier of day-old chicks, who undertook to hatch Light Sussex chicks for us at regular intervals, and so were enabled to keep the experiments running consecutively during summer and winter throughout the several years of this work. The chicks were not of the same parentage, but were hatched from eggs obtained from the same breeder except at times of scarcity when eggs from

another breeder were also used. According to the dictum of Osborne *et al.* [1919] experiments on the nutritive values of proteins should be made with large numbers of animals. It was expected that 10 or 12 chickens would be sufficient, but owing to deaths in the early weeks of the experiments and in order to have enough birds at the end of 20 or 25 weeks, 15 one day-old chicks were generally started. The losses can be seen from Table I. The chicks were kept in groups as it was not possible for us to rear single birds in separate cages with the number that would be needed in each test.

The groups were started in foster-mothers, shifted to small houses at the age of 6 or 8 weeks and then to larger houses. All the appliances were the same as previously described [1923] and kept on the roof of the building. At times if the weather were cold, or if the chicks were not large enough to move from the foster-mothers, the first rearing was carried out in a room of the laboratory in large boxes in which an electric light was suspended to keep the birds warm. Elaborate plant is not necessary for starting the rearing of chicks. The birds need only warmth and suitable food. Feeding was started at 48 hours after hatching.

The birds were weighed individually every week. At the end of the experiments, when the sexes were known, the weights of the surviving cocks and hens could be separated and the mean weights of each sex could be calculated for each week. It was seldom that the numbers of each sex in a group were the same. The separate figures are necessary for constructing growth curves. With reference to food consumption, it is not possible in mixed groups to separate the food eaten by the two sexes. Cocks not only grow faster and more than hens but they eat more food. We have been unable to test whether the increase in weight of each sex is the same for the same amount of food eaten, and from our data it is not possible to make any computation of the consumption of the separate sexes. It has been necessary to assume that increase in relation to food consumption is the same for both sexes. The average increase of both sexes and of those birds surviving to the end of the experiments is given in Table II.

Diet.

The first consideration in compounding a suitable diet for feeding groups of 10–15 chickens over periods of 15–25 weeks in a series of experiments which would continue during several years was the provision of vitamins in adequate amounts. Our previous experiments had been carried out to ascertain these requirements.

Vitamins A and D. It was found [1927] that 0·5 % of cod-liver oil supplied enough vitamins A and D, though chicks could be reared on 0·1 %. The diets were therefore made to contain 1 %; in some experiments 2 or even 3 % was used, as it seemed during the trials that the smaller amount was insufficient. The cod-liver oil was procured from the same source as in the original tests.

Preparations of activated ergosterol and carotene were not available at the beginning of this work, and the requirements of birds for these compounds have not yet been determined. There should be no objection to the use of cod-liver oil.

Small amounts of these vitamins may have been introduced with the protein foodstuff. The addition would be most marked in the experiments with fresh egg-yolk, but other experiments with egg-yolk proteins freed from fat gave very similar results, so that the extra vitamins A and D may be considered to have exercised very little influence upon the increase in weight.

Vitamin B_1 or the vitamin B complex. Vitamin B_1 preparations were not procurable at the start of these experiments. Special concentrates made from

yeast by the method of Kinnersley and Peters would have been required in such large quantities that their preparation was not a practicable proposition, and moreover each batch would have needed testing for its content of vitamin B_1.

The requirements of chickens for vitamin B_1 had been investigated by us [1923] in terms of marmite, and we had determined the comparative vitamin B_1 values of dried yeast and wheat germ [1927; 1931]. The amounts that would be needed on a pure starch diet were so high that the use of either of these materials was excluded, as it entailed the addition of a good deal of protein which might alter the value of the protein to be tested.

The alternative for the supply of sufficient vitamin B_1 was the use of whole grain. We had found that pigeons could rear their young on diets containing from 75 to 90 % of whole grain [1927], and though chicks could not be satisfactorily reared on oatmeal without extra vitamin B_1 [1923] it was expected from the close similarity of different birds that chicks could be reared on certain whole grains if these formed about 90 % of the diet.

The results of experiments with whole grain would be more in conformity with the diets of everyday life, with which outlook our work was planned.

In testing the proteins of yeast, wheat germ and pulses extra vitamin B_1 was introduced. The change did not produce an inordinate amount of growth. The extra quantity rather improved the general health of the birds.

Vitamin C. We were the first to show that birds did not require vitamin C in their diet [1923].

Carbohydrate. Birds are normally eaters of grain. Starch is thus their standard form of carbohydrate. With the use of grain as source of vitamin B_1 starch became the basal carbohydrate in these experiments.

The choice of grain in the first experiments was maize on account of its known inadequate types of protein, but in all subsequent experiments wheat was used. Wheat is preferred by small chicks to the hard material of maize. It also conforms to the common use of wheat as basal carbohydrate food of man.

Fat. Chicks had previously been reared by us [1927] on diets without additional fat beyond the cod-liver oil used for supplying vitamins A and D. There was no reason to introduce further fat into the diets. Only in the experiments with egg-yolk and meat-meal would a proportion of extra fat be introduced. Additional experiments were made with fat-extracted meat-meal and egg-yolk and no special difference in the result was observed.

Mineral salts. In all our previous work we have used fish-meal as basal protein since mineral salts were supplied at the same time. We preferred to continue with this salt mixture rather than use the mixture of Osborne and Mendel or that of McCollum. In the earlier tests fish-meal was ashed and the ash mixed with the other parts of the food, but later an artificial mixture corresponding with the analysis of the ash was used. In the comparison of fish-meal with wheat germ the fish-meal diet was made equivalent by adding an artificial ash corresponding with the ash of wheat germ. As the amount of mineral salts introduced with fish-meal was approximately 1 %, 1 % or occasionally 2 % of mineral salts was incorporated in the diets.

Proteins. Since the chief object of these experiments was the determination of the relative values of proteins in their natural form in foodstuffs, it was not necessary to separate the protein from the available material. In their natural form several different proteins may be present together and they are eaten as a mixture and not separately. In many cases like meat and fish the separation is not possible.

Fish. The type of fish-protein was that contained in a commercial fish-meal prepared from white fish and containing about 3 % of fat.

Meat. The material, meat-meal, consisted of the residue after preparation of meat extract. It was a fine brown powder containing about 5 % of fat. It was kindly given to us for these experiments by the late Dr M. J. Rowlands.

Blood. Commercial blood-meal consisting of dark brown almost black granules was used.

Lactalbumin. The preparation of enough lactalbumin from milk for these experiments was not possible at the same time as the carrying out of the feeding tests and we tried to obtain a good commercial material. The appearance of commercial lactalbumin was very unappetising. It consisted of hard dark brown lumps of various sizes mixed with some powder. For feeding the lumps had to be passed through a grinder. The results of the experiments show the poor quality of this material. They are not comparable with the high value of lactalbumin found by Osborne and Mendel.

Caseinogen. Several varieties of caseinogen are available commercially. "Light white casein" of the B.D.H. was chosen as it seemed to contain some factor which is not present in the vitamin-free preparations.

Whole dried milk, or dried skimmed milk, was not used as this material would have introduced lactose and phosphates and so upset the general composition of the diets.

Egg-white and egg-yolk. The whole white and the whole yolk of hard-boiled fresh eggs were used, the parts being separated by hand as completely as possible and put through a mincer to break up into smaller pieces. It was desired to test these parts separately, but rationally, in agreement with the other tests, they should have been tried together.

On account of the high content of water in these materials which entailed using less grain in the diets than in the other experiments, the results may belong to the next series (see below and Table I under protein ratio). In order to have a diet more comparable with the others, experiments were also made with dried egg-white and dried egg-yolk from which the fat was extracted. The fresh hard-boiled whites were broken up, spread out in a basin and dried in a steam-oven. The hard-boiled yolks were spread out on plates to dry and then extracted with ether in an extractor. The residue was exposed to the air for the ether to evaporate.

Yeast. Commercial dried yeast was kindly placed at our disposal by the Marmite Food Extract Co., Ltd.

Wheat germ. We were supplied with dried wheat germ, Bemax, through the kindness of the late Dr M. J. Rowlands, by Vitamins, Ltd.

Peas, lentils. The ordinary forms of split brown peas and split red lentils as ordinarily obtainable were used. They were ground up for feeding to the chicks.

Soya bean. Commercial powdered soya bean-meal from which the fat had been extracted was procured. It consisted of a pale brown powdery material.

Bean. It was more convenient to use a commercial form of bean-meal than haricot or butter beans. It formed a mottled powder of a granular nature.

Alfalfa. The commercial material consisted of a fine pale green powder.

Grass. Dried grass prepared by Imperial Chemical Industries, Ltd., was placed at our disposal by Dr H. J. Page of the Agricultural Station at Jealotts Hill. It consisted of fine particles and long fibres difficult to break up into smaller pieces.

Gluten. The commercial gluten consisted of a fine almost white powder.

Quantity of protein. Previous work on the determination of the biological values of proteins seems to insist on including the protein in increasing quantities at so-called "different levels of intake." Our experiments were therefore designed in a similar way. In our preliminary experiments with maize we used 5, 10, 15 and 20 % of fish-meal. These amounts correspond with 3·05, 4·57 and 6·1 % of protein. The amount of protein in the materials was estimated from the nitrogen content multiplied by the factor 6·25. The quantity containing 3·05, or 4·57, or 6·1 was then calculated and used in the feeding trials. The protein values of the different materials were:

	%		%
Fish-meal	57·5	Barley	8·66
Meat-meal	59·9	Oats	7·72
Fat-free meat-meal	89·6	Oatmeal	11·52
Blood-meal	79·8	Maize	7·56
Caseinogen	77·5	Rye	10·56
Lactalbumin	56·4	Buckwheat	12·49
Fresh egg-white	10·7	Brown rice	7·39
Fresh egg-yolk	15·5	Millet	10·63
Dried egg-white	74·6	Dari	8·14
Dried yeast	47·8	Split peas	19·48
Dried wheat germ	30·7	Split lentils	22·71
Alfalfa	12·4	Bean-meal	23·99
Dried grass	12·4	Soya bean-meal	43·4
Wheat	9·04*		

* This is the mean value of the various supplies. The value varied from 7·0 to 10·2 %. The low values were due to condensation of moisture on the grain during transit.

Mixing of the foodstuffs. With the quantity of protein material thus fixed, with the addition of 1 % of mineral salts and 1 or 2 % of cod-liver oil, the amount of grain in the diet was the quantity to total 100. The grain, at first maize, then wheat, was passed through a mill and ground to a fine or coarse powder according to the size of the chicks to be fed, mixed with the mineral salts, and protein material and then stirred up thoroughly with the cod-liver oil which was shaken with water to make a fine emulsion. The chickens were thus fed with a mash throughout the period of the experiment. The food was given *ad lib.* Fresh supplies were made daily or every other day according to requirements and fed only when the previous lot was eaten. A record of all food supplied was kept.

Using grain as source of carbohydrate and vitamin B_1, and adding mineral salts, cod-liver oil, and the requisite quantity of protein the diet was as simple as possible.

The diets with the dates of the experiments, number of birds, and amounts of protein and the protein ratio are given in Table I A to E under the headings of maize series, series 0, series 1, series 2, series 3. Series 0 represents the experiments with cereals and pulses, series 1, 2 and 3 the experiments with 3·05, 4·57 and 6·1 % of protein respectively with wheat.

The results of the experiments are given in Table II A to E in which the food consumption, the consumption of protein, and the increase in weight are recorded.

Table I. *Diets. Cereals and pulses.*

A. *Series 0.*

Seed	g.	Cod-liver oil g.	Salts g.	Protein g.	Experiment Dates	Number of birds	Cock	Hen
Maize	98	1·0	1·0	7·4	15. x. 25–30. vi. 26	7	2	0
	97	2·0	1·0	7·3	17. iii. 30–21. vi. 30	15	2	0
Wheat	98	1·0	1·0	8·9	27. vi. 27–19. xii. 27	7	3	3
	98	1·0	1·0	8·9	1. ii. 30–25. vii. 30	15	1	4
Barley	98	1·0	1·0	8·4	20. vii. 27–11. i. 28	10	5	4
	96	3·0	1·0	8·4	2. v. 30–24. x. 30	15	3	4
Rye	98	1·0	1·0	10·3	22. vii. 28–13. i. 29	14	0	5
	97	2·0	1·0	10·2	21. ii. 30–15. viii. 30	15	0	2
Oatmeal	98	1·0	1·0	11·3	12. iv. 29–12. ix. 29	15	2	2
	97	2·0	1·0	11·2	22. ii. 30–15. viii. 30	15	3	3
Oats	96	3·0	1·0	7·4	3. v. 30–24. x. 30	15	4	4
Buckwheat	98	1·0	1·0	12·2	7. ix. 28–28. ii. 29	15	5	2
	98	1·0	1·0	12·2	12. iv. 29–3. x. 29	15	8	3
Millet	98	1·0	1·0	10·4	7. ix. 28–7. ii. 29	15	1	3
	97	2·0	1·0	10·3	9. iv. 30–22. vii. 30	15	0	1
Dari	98	1·0	1·0	8·0	23. vii. 28–13. i. 29	15	1	3
	97	2·0	1·0	7·9	9. iv. 30–29. vii. 30	15	1	0
Brown rice	98	1·0	1·0	7·2	23. ix. 29–19. x. 29	15	1	0
	97	2·0	1·0	7·2	17. iii. 30–30. vi. 30	15	1	0
Dried green peas	98	1·0	1·0	21·9	14. x. 27–20. i. 28	11	1	1
Split peas	97	2·0	1·0	18·9	11. v. 31–1. xi. 31	15	0	2
Split lentils	97	2·0	1·0	22·0	11. v. 31–1. xi. 31	15	2	1
Bean-meal	97	2·0	1·0	23·3	17. i. 31–27. iii. 31	15	2	0
Soya bean-meal	97	2·0	1·0	42·1	17. i. 31–5. vi. 31	15	0	3

Table I (*cont.*).

B. *Maize series.*

			Cod-liver oil		Proteins					Number of birds	
	g.	Maize g.	g.	Salts g.	Added g.	Maize g.	Total g.	Ratio	Experiment Dates	Cock	Hen
Fish-meal (1)	5·0	94·0	1·0	—	2·9	7·1	10·0	1:2·45	28. i. 26–21. vii. 26	12	5 4
Fish-meal (2)	10·0	89·0	1·0	—	5·8	6·7	12·5	1:1·15	29. v. 26–12. xi. 26	10	7 1
Fish-meal (3)	15·0	84·0	1·0	—	8·6	6·3	14·9	1:0·73	16. vii. 26–16. xii. 26	9	2 2
Fish-meal (4)	20·0	79·0	1·0	—	11·5	6·0	17·5	1:0·52	30. viii. 26–30. i. 27	13	5 4
Dried yeast (1)	6·0	91·0	1·0	2·0	2·9	6·9	9·8	1:2·40	20. iv. 28–11. x. 28	10	1 1
Dried wheat germ (3)	28·0	69·0	1·0	2·0	8·6	5·2	13·8	1:0·60	28. ii. 28–16. vii. 28	10	4 1
Dried wheat germ (2)	18·6	78·4	1·0	2·0	5·7	5·9	11·6	1:1·04	23. iii. 28–10. viii. 28	11	1 6

C. *Series 1.*

			Cod-liver oil		Proteins					Number of birds	
	g.	Wheat g.	g.	Salts g.	Added g.	Wheat g.	Total g.	Ratio	Experiment Dates	Cock	Hen
Fish-meal	5·3	93·2	1·0	0·5	3·05	8·43	11·48	1:2·76	25. ii. 29–14. vii. 29	15	5 3
Meat-meal	5·1	92·9	1·0	1·0	3·05	8·40	11·45	1:2·75	5. xi. 29–28. iv. 30	15	0 7
Fat-free meat-meal	3·4	94·6	1·0	1·0	3·05	8·55	11·60	1:2·80	25. ii. 29–14. vii. 29	15	1 6
Blood-meal	3·8	94·2	1·0	1·0	3·03	8·52	11·55	1:2·81	4. xi. 29–31. iii. 30	15	2 1
Caseinogen	3·9	93·1	2·0	1·0	3·02	8·42	11·44	1:2·79	1. x. 30–25. iii. 31	15	2 4
Lactalbumin	5·4	91·6	2·0	1·0	3·05	8·28	11·33	1:2·71	12. xi. 30–5. v. 31	14	2 4
Dried egg-white	4·1	92·9	2·0	1·0	3·06	8·40	11·46	1:2·75	3. xii. 30–27. v. 31	15	4 8
Dried egg-yolk	3·9	93·1	2·0	1·0	3·02	8·42	11·44	1:2·79	22. x. 30–15. iv. 31	14	5 4
Fresh egg-white	28·5	69·5	1·0	1·0	3·05	6·28	9·33	1:2·06	16. x. 29–4. iii. 30	15	2 4
Fresh egg-yolk	19·7	78·3	1·0	1·0	3·05	7·08	10·13	1:2·32	16. x. 29–5. iii. 30	12	4 4
Dried yeast	6·4	91·6	1·0	1·0	3·06	8·28	11·34	1:2·71	21. xii. 29–9. v. 30	15	5 5
Dried wheat germ	10·0	88·0	1·0	1·0	3·07	7·96	11·03	1:2·59	23. x. 28–15. iv. 29	15	5 6
Split peas	15·6	81·4	2·0	1·0	3·04	7·36	10·40	1:2·42	18. iv. 31–9. x. 31	15	9 6
Split lentils	13·4	83·6	2·0	1·0	3·04	7·56	10·60	1:2·49	18. iv. 31–9. x. 31	15	1 5
Bean-meal	12·7	84·3	2·0	1·0	3·05	7·62	10·67	1:2·50	2. iii. 31–23. viii. 31	15	2 3
Soya bean-meal	7·0	90·0	2·0	1·0	3·04	8·14	11·18	1:2·68	2. iii. 31–23. viii. 31	15	2 2
Alfalfa	24·7	72·3	2·0	1·0	3·06	6·54	9·60	1:2·14	24. vi. 31–16. xii. 31	15	2 7
Dried grass	24·7	72·3	2·0	1·0	3·06	6·54	9·60	1:2·14	24. vi. 31–16. xii. 31	15	2 4

Table I (*cont.*).

D. *Series 2.*

	g.	Wheat g.	Cod-liver oil g.	Salts g.	Proteins: Added g.	Proteins: Wheat g.	Proteins: Total g.	Ratio	Experiment Dates	Number of birds	Cock	Hen
Fish-meal	7·9	90·4	1·0	0·7	4·54	8·17	12·71	1 : 1·80	3. ii. 29–1. vii. 29	13	5	3
Meat-meal	6·8	91·2	1·0	1·0	4·07	8·24	12·31	1 : 2·20	27. v. 29–20. xi. 29	15	3	5
Fat-free meat-meal	5·1	92·3	1·0	1·0	4·57	8·34	12·91	1 : 1·61	2. ii. 29–1. vii. 29	15	4	4
Blood-meal	5·1	92·9	1·0	1·0	4·07	8·40	12·47	1 : 2·06	29. v. 29–20. xi. 29	15	5	5
Caseinogen	5·9	91·1	2·0	1·0	4·57	8·24	12·81	1 : 1·80	25. vii. 30–19. xii. 30	15	3	7
Lactalbumin	8·1	88·9	2·0	1·0	4·57	8·04	12·61	1 : 1·76	25. vii. 30–9. i. 31	15	7	6
Dried egg-white	6·1	90·9	2·0	1·0	4·55	8·22	12·77	1 : 1·80	28. viii. 30–11. ii. 31	15	2	9
Dried egg-yolk	5·9	91·1	2·0	1·0	4·57	8·24	12·81	1 : 1·80	20. viii. 30–7. i. 31	15	4	11
Fresh egg-white	28·5	69·5	1·0	1·0	3·05	6·28	9·33	1 : 2·06	16. x. 29–5. iii. 30	14	2	4
Fresh egg-yolk	19·7	78·3	1·0	1·0	3·05	7·08	10·13	1 : 2·32	16. x. 29–5. iii. 30	12	4	4
Dried yeast (*a*)	9·5	87·5	1·0	2·0	4·54	7·91	12·45	1 : 1·74	14. v. 28–1. x. 28	13	7	4
(*b*)	9·56	88·44	1·0	1·0	4·57	7·99	12·56	1 : 1·75	10. i. 30–16. vi. 30	15	2	4
Dried wheat germ (*a*)	15·0	81·0	2·0	2·0	4·60	7·32	11·92	1 : 1·60	30. vi. 28–16. xi. 28	10	4	2
(*b*)	15·0	82·4	1·0	1·6	4·60	7·45	12·05	1 : 1·61	15. xi. 28–8. v. 29	15	7	5
Gluten	6·2	91·8	1·0	1·0	4·57	8·30	12·87	1 : 1·81	13. i. 30–17. ii. 30	15	0	0
Split peas	23·5	73·5	2·0	1·0	4·58	6·65	11·23	1 : 1·45	25. iii. 31–16. ix. 31	15	5	4
Split lentils	20·1	76·9	2·0	1·0	4·57	6·95	11·52	1 : 1·52	25. iii. 31–16. xi. 31	15	1	2
Bean-meal	23·3	73·7	2·0	1·0	4·57	6·66	11·23	1 : 1·46	24. xii. 30–17. vi. 31	15	3	3
Soya bean-meal	10·5	86·5	2·0	1·0	4·56	7·82	12·38	1 : 1·71	24. xii. 30–17. vi. 31	9	1	4
Dried alfalfa	37·0	60·0	2·0	1·0	4·58	5·42	10·00	1 : 1·18	1. vi. 31–23. xi. 31	15	1	6
Dried grass	37·0	60·0	2·0	1·0	4·58	5·42	10·00	1 : ·18	1. vi. 31–23. xi. 31	15	1	3

E. *Series 3.*

Fish-meal	11·0	88·0	1·0	—	6·33	7·96	14·29	1 : 1·26	22. iii. 28–10. viii. 28	12	4	7
Meat-meal	10·2	87·8	1·0	1·0	6·11	7·94	14·05	1 : 1·30	29. xi. 29–18. iv. 30	15	1	4
Fat-free meat-meal	6·8	90·1	1·0	2·1	6·09	8·15	14·24	1 : 1.33	20. iii. 29–7. viii. 29	15	2	4
Blood-meal	7·6	90·4	1·0	1·0	6·06	8·17	14·23	1 : 1·35	20. xii. 29–28. ii. 30	15	2	2
Caseinogen (*a*)	7·87	89·13	2·0	1·0	6·10	8·06	14·16	1 : 1·32	10. xi. 30–4. ii. 31	15	1	2
(*b*)	7·80	87·20	4·0	1·0	6·05	7·88	13·93	1 : 1·32	3. xii. 30–22. iv. 31	15	0	1

Table I. E. *Series 3 (cont.).*

		Proteins							Experiment Dates	Number of birds	
		Wheat g.	Cod-liver oil g.	Salts g.	Added g.	Wheat g.	Total g.	Ratio		Cock	Hen
Lactalbumin	g. 10·8	86·2	2·0	1·0	6·09	7·79	13·88	1 : 1·28	10. ix. 31–28. i. 32	3	5
Dried yeast	12·6	83·4	2·0	2·0	6·02	7·54	13·56	1 : 1·25	30. vi. 28–23. xi. 28	3	3
Dried wheat germ	(*a*) 20·0	77·0	1·0	2·0	6·14	6·96	13·10	1 : 1·13	24. xii. 27–7. v. 28	3	5
	(*b*) 20·0	76·0	2·0	2·0	6·14	6·87	13·01	1 : 1·12	8. vi. 28–26. x. 28	7	9
	(*c*) 20·0	76·9	1·0	2·1	6·14	6·95	13·09	1 : 1·13	20. iii. 29–7. viii. 29	8	6
Gluten	8·5	88·5	1·0	2·0	6·26	8·00	14·26	1 : 1·28	16. i. 28–16. vii. 28	2	3

Table II. *Food consumption and increase in weight per bird.*

A. *Series 0.*

Figures represent g.

In weeks	(1) Wheat	Cod-liver oil	Salts	*Wheat protein*	Weight increase	(2) Wheat	Cod-liver oil	Salts	*Wheat protein*	Weight increase
5	293·4	3·3	3·3	26·5	45	208·3	4·0	2·2	18·8	29
10	824·8	8·9	8·9	74·6	128	753·9	15·1	7·8	68·1	100
15	2003·1	21·0	21·0	181·1	291	1838·9	37·4	19·0	166·2	237
20	3767·1	39·0	39·0	340·5	522	2905·9	59·4	30·0	282·7	341
25	5923·1	61·0	61·0	535·4	846	4069·9	83·4	42·0	367·9	463
	(1) Barley			*Barley protein*		(2) Barley			*Barley protein*	
5	313·6	3·2	3·2	27·0	48	307·6	9·6	3·5	26·4	62
10	1179·3	12·0	12·0	101·4	183	734·3	22·8	7·9	63·2	131
15	2747·2	28·1	28·1	236·3	445	1816·4	56·5	19·3	156·2	309
20	5491·3	56·0	56·0	472·2	866	3399·4	106·0	35·8	292·3	557
25	9411·3	96·0	96·0	809·4	1346	5593·6	174·7	58·7	481·1	886
	(1) Rye			*Rye protein*		(2) Rye			*Rye protein*	
5	283·1	2·9	2·9	29·9	40	226·8	4·6	2·5	23·9	21
10	936·5	9·5	9·5	98·9	133	698·2	14·4	7·3	73·7	81
15	2190·9	22·3	22·3	231·4	237	1506·8	31·1	15·6	159·1	183
20	3366·9	34·3	34·3	355·5	329	2476·8	51·1	25·6	261·5	258
25	5228·9	53·3	53·3	552·2	615	3640·8	75·1	37·6	384·5	378
	(1) Buck-wheat			*Buck-wheat protein*		(2) Buck-wheat			*Buck-wheat protein*	
5	450·5	4·6	4·6	56·3	91	376·0	3·8	3·8	47·0	75
10	1889·5	19·3	19·3	236·0	313	1418·4	14·4	14·4	177·2	242
15	4269·5	43·6	43·6	533·3	717	3235·7	33·1	33·1	404·1	613
20	7629·5	77·9	77·9	952·9	1267	6122·1	62·4	62·4	764·6	1111
25	12389·5	126·5	126·5	1547·4	1919	9436·2	96·0	96·0	1178·6	1489

Table II. A. *Series 0 (cont.).*

In weeks	(1) Oat-meal	Cod-liver oil	Salts	*Oatmeal protein*	Weight increase	(2) Oat-meal	Cod-liver oil	Salts	*Oatmeal protein*	Weight increase
5	209·5	2·2	2·2	24·1	53	219·8	4·6	2·3	25·3	48
10	797·5	8·2	8·2	91·9	207	615·6	12·9	6·5	70·9	163
15	1659·9	17·0	17·0	191·2	417	1491·1	30·9	15·6	171·8	307
20	2992·7	30·6	30·6	344·8	642	2542·0	52·5	26·5	292·8	482
25	—	—	—	—	—	4134·5	94·1	42·2	476·3	627

	Oats			*Oats protein*	
5	355·0	11·0	3·7	27·4	66
10	1057·7	33·1	11·1	81·7	139
15	2161·7	67·6	22·6	167·0	308
20	3481·7	108·8	36·3	269·0	492
25	5761·7	180·0	60·0	445·1	843

	(1) Maize			*Maize protein*		(2) Maize			*Maize protein*	
5	214·7	2·1	2·1	16·2	20	159·7	3·2	1·6	12·1	8
10	658·9	6·7	6·7	49·8	77	368·0	7·5	3·7	27·8	34
15	1482·1	15·1	15·1	112·0	183	—	—	—	—	—
20	2971·7	30·3	30·3	224·7	369	—	—	—	—	—
25	4476·0	45·7	45·7	338·4	514	—	—	—	—	—
30	6240·0	63·7	63·7	471·7	746	—	—	—	—	—
35	8347·0	85·2	85·2	631·0	1044	—	—	—	—	—

	(1) Millet			*Millet protein*		(2) Millet			*Millet protein*	
5	220·6	2·3	2·3	23·4	15	185·4	3·9	1·8	19·7	14
10	556·9	5·7	5·7	59·2	46	524·7	10·8	5·4	55·8	34
15	1213·5	12·4	12·4	129·0	112	977·3	20·1	10·1	103·9	55
20	2316·0	23·5	23·5	246·2	231	—	—	—	—	—

	(1) Dari			*Dari protein*		(2) Dari			*Dari protein*	
5	244·5	2·6	2·6	19·9	30	158·0	3·3	1·6	12·9	12
10	668·3	6·9	6·9	54·4	64	487·8	9·7	4·8	39·7	30
15	1452·2	15·0	15·0	118·2	101	1069·8	21·7	10·8	87·1	60
20	2750·7	28·2	28·2	223·9	185	—	—	—	—	—
25	4343·2	44·3	44·3	353·5	324	—	—	—	—	—

	(1) Brown rice			*Rice protein*		(2) Brown rice			*Rice protein*	
5	123·2	1·2	1·2	9·1	12	219·9	4·5	2·3	16·2	22
10	319·2	3·2	3·2	23·6	43	486·2	10·0	5·0	35·9	30
15	—	—	—	—	—	1068·2	22·0	11·0	78·9	85

	Split peas			*Peas protein*		Lentils			*Lentils protein*	
5	197·6	4·1	2·0	38·5	38	170·9	3·7	1·7	38·8	34
10	908·9	18·8	9·3	86·6	166	509·7	10·7	5·2	115·7	88
15	1975·9	40·8	20·3	384·9	399	1169·3	24·3	12·0	265·6	217
20	3624·9	74·8	37·3	706·1	699	2149·0	44·5	22·1	488·0	390
25	6049·9	124·8	62·3	1178·5	1041	3442·4	71·0	35·5	781·8	596

	Bean-meal			*Bean-protein*		Soya bean-meal			*Soya protein*	
5	436·5	9·0	4·5	104·7	61	358·5	7·4	3·7	155·6	71
10	1406·5	29·0	14·5	337·4	251	1153·9	23·8	11·9	500·8	182
15	—	—	—	—	—	2899·9	59·8	29·9	1258·6	410
20	—	—	—	—	—	4751·0	98·0	49·0	2061·9	675

Table II (*cont.*).

B. *Maize series.*

In weeks	Maize	Fish-meal	Cod-liver oil	Salts	Protein: Maize	Protein: *Fish*	Protein: Total	Weight increase
		(1)						
5	274·2	14·5	2·9	—	20·7	8·3	29·0	62
10	1095·7	58·3	11·6	—	82·8	33·5	116·3	244
15	2547·5	135·5	27·1	—	192·6	77·9	270·5	547
20	4385·8	233·2	46·7	—	331·6	134·1	465·7	879
25	6767·2	359·8	72·0	—	511·6	206·9	718·5	1212
		(2)						
5	394·3	44·4	4·5	—	29·8	25·5	55·3	106
10	1284·4	144·4	14·6	—	97·1	83·0	180·1	399
15	3264·6	366·9	36·8	—	246·8	211·0	457·8	1078
20	6468·6	726·9	72·8	—	489·0	418·0	907·0	1936
24	9939·6	1116·9	111·8	—	741·4	642·2	1383·6	2645
		(3)						
5	401·3	71·7	4·8	—	30·3	41·2	71·5	180
10	1633·4	278·6	19·6	—	123·5	160·2	283·7	657
15	4564·0	802·0	54·4	—	345·0	461·2	806·2	1669
20	8260·0	1462·0	98·4	—	624·5	840·7	1465·2	2043
		(4)						
5	381·0	96·5	4·8	—	28·8	55·5	84·3	170
10	2134·8	540·5	27·0	—	161·4	310·8	472·2	799
15	4741·8	1200·5	60·0	—	358·5	690·3	1049·8	1687
20	8059·8	2040·5	94·0	—	609·3	1173·3	1782·6	2297
		Dried wheat germ				*Wheat germ*		
		(2)						
5	277·1	65·8	3·5	7·0	21·0	20·2	41·2	75
10	874·9	207·6	11·1	22·3	66·1	63·7	129·8	261
15	2302·9	546·3	29·4	58·7	174·1	167·7	341·8	597
20	3422·9	811·9	43·8	87·2	258·8	249·5	508·3	1008
		(3)						
5	249·9	102·8	3·7	7·2	18·9	31·6	50·5	109
10	1168·9	410·8	14·7	29·2	88·4	126·1	214·5	367
15	2647·3	1015·6	48·3	72·4	200·1	324·1	524·2	861
20	4891·3	1939·6	114·3	138·4	369·8	595·5	965·3	1569
		Dried yeast (1)				*Yeast*		
5	248·3	16·4	2·7	5·5	18·8	7·8	26·6	32
10	1021·8	67·4	11·2	22·5	77·3	32·2	109·5	139
15	2295·8	151·4	25·2	50·5	173·6	72·4	246·0	339
20	3387·8	223·4	37·2	74·5	256·1	106·8	362·9	452
25	5389·8	355·4	59·2	118·5	407·5	169·9	577·4	612

C. *Series 1.*

Figures represent g.

In weeks	Wheat	Fish-meal	Cod-liver oil	Salts	Protein: Wheat	Protein: *Fish*	Protein: Total	Weight increase
5	320·5	18·2	3·4	1·7	29·0	10·5	39·5	81
10	1499·9	85·3	16·1	8·0	135·6	49·1	184·7	348
15	3363·9	191·3	36·1	18·0	304·1	110·0	414·1	779
20	5693·9	323·8	61·1	30·5	514·7	186·2	700·9	1395
		Meat-meal				*Meat*		
5	354·7	19·4	3·7	3·7	32·1	11·6	43·7	81
10	1425·1	78·2	15·1	15·1	128·8	46·8	175·6	319
15	3415·7	187·6	36·6	36·6	308·8	112·4	421·2	787
20	6733·7	369·6	72·3	72·3	608·7	221·4	830·1	1374

Table II. C. *Series 1 (cont.).*

In weeks	Wheat	Fat-free meat-meal	Cod-liver oil	Salts	Protein: Wheat	Protein: *Meat*	Protein: Total	Weight increase
5	398·1	14·3	4·2	4·2	36·0	12·8	48·8	84
10	1602·5	57·7	16·9	16·9	145·1	51·7	196·8	314
15	3494·7	125·9	37·1	37·1	315·9	112·8	428·7	658
20	5528·4	202·4	59·6	59·6	499·8	181·3	681·1	1042
		Blood-meal				*Blood*		
5	344·9	13·9	3·7	3·7	31·2	11·1	42·3	74
10	1448·4	58·4	15·4	15·4	130·9	46·6	177·5	254
15	3388·8	136·7	36·0	36·0	306·3	109·1	415·4	612
20	6342·5	256·9	96·7	67·8	573·4	205·0	778·4	1226
		Caseinogen				*Caseinogen*		
5	380·5	16·0	8·1	4·1	34·4	12·4	46·8	113
10	1494·4	62·6	32·0	16·1	135·1	48·5	183·6	393
15	3555·9	148·9	76·3	38·3	321·4	115·4	436·8	919
20	6969·7	291·9	149·5	74·7	630·1	226·2	856·3	1536
25	10538·7	444·7	226·1	112·8	952·7	344·6	1297·3	2060
		Lact-albumin				*Lact-albumin*		
5	159·9	9·1	3·5	1·8	14·5	5·1	19·6	33
10	536·6	31·2	11·9	5·9	48·5	17·6	66·1	102
15	1604·6	93·2	35·2	17·6	145·1	52·6	197·7	336
20	3589·2	210·2	78·4	39·2	324·5	118·5	443·0	676
25	6031·9	354·2	131·7	65·8	545·3	199·8	745·1	1106
		Dried egg-white				*Egg-white*		
5	188·1	8·4	4·1	2·0	17·0	6·3	23·3	48
10	924·3	40·9	19·8	9·9	83·6	30·5	114·1	236
15	2627·6	116·2	56·5	28·2	237·5	86·7	324·2	618
20	5104·9	225·5	109·8	54·9	461·5	168·2	629·7	1202
25	8511·2	375·8	183·1	91·6	769·4	280·4	1049·8	1711
		Dried egg-yolk				*Egg-yolk*		
5	222·6	9·3	4·8	2·4	20·1	7·2	27·3	67
10	913·6	39·1	20·1	10·0	82·6	30·3	112·9	269
15	2578·2	111·5	57·4	28·5	233·1	86·4	319·5	711
20	5117·2	218·0	111·9	55·9	462·6	168·9	631·5	1394
25	8214·9	347·9	178·3	89·3	742·6	269·6	1012·2	1941
		Fresh egg-yolk				*Egg-yolk*		
5	241·8	61·0	3·1	3·1	22·9	9·5	32·4	75
10	1168·4	294·1	14·9	14·9	105·6	45·6	151·2	287
15	2734·4	688·1	34·9	34·9	247·2	106·7	353·9	718
20	4794·8	1206·7	61·2	61·2	433·4	187·0	620·4	1143
		Fresh egg-white				*Egg-white*		
5	403·3	165·4	5·8	5·8	36·5	17·7	54·2	130
10	1793·3	735·4	25·8	25·8	162·1	78·7	240·8	541
15	4565·3	1872·9	65·5	65·5	412·7	200·4	613·1	1216
20	7894·0	3238·0	113·4	113·4	713·6	346·5	1060·1	1780

Table II. C. *Series 1 (cont.).*

In weeks	Wheat	Dried yeast (*a*)	Cod-liver oil	Salts	Protein: Wheat	Protein: *Yeast*	Protein: Total	Weight increase
5	323·1	22·7	3·6	3·6	29·2	10·9	40·1	70
10	1435·4	100·4	15·7	15·7	129·8	48·0	177·8	315
15	3267·5	228·3	35·8	35·8	295·4	109·1	404·5	684
20	5828·9	407·7	71·0	63·8	526·9	194·9	721·8	1191
25	9205·8	646·2	145·5	101·1	832·2	309·0	1141·2	1637
		Dried wheat germ				*Wheat germ*		
5	330·0	37·7	3·8	5·5	30·8	11·6	42·4	76
10	1301·5	148·2	14·8	16·5	117·7	45·5	163·2	228
15	2885·5	328·2	32·8	34·5	260·8	100·8	361·6	558
20	5349·5	608·2	60·8	62·5	483·6	186·7	670·3	983
25	8341·5	948·2	94·8	96·5	754·1	291·1	1045·2	1610
		Split peas				*Peas*		
5	206·3	39·4	5·1	2·5	18·6	7·7	26·3	65
10	683·7	130·9	16·9	8·3	61·8	25·5	87·3	179
15	1552·0	297·3	38·2	19·0	140·3	58·0	198·3	418
20	2908·6	557·3	71·6	35·6	262·9	108·7	371·6	702
25	4536·5	869·3	111·6	55·5	410·1	169·5	579·6	1063
		Split lentils				*Lentils*		
5	196·2	31·5	4·7	2·3	17·7	7·2	24·9	58
10	642·2	103·0	15·3	7·6	58·1	23·4	81·5	181
15	1358·6	217·8	33·4	16·0	122·8	49·5	172·3	278
20	2343·8	375·7	56·0	27·7	211·9	85·3	297·2	530
25	3876·5	621·4	92·7	46·0	350·4	141·1	491·5	786
		Bean-meal				*Bean*		
5	282·2	42·6	6·7	3·3	25·5	10·3	35·8	76
10	1013·5	152·7	24·1	12·0	91·6	36·6	128·2	219
15	2218·0	334·2	52·6	26·5	200·5	80·2	280·7	467
20	3863·9	582·1	91·5	46·0	349·3	139·7	489·0	828
25	5887·1	886·9	139·5	70·0	532·2	212·8	745·0	1242
		Soya bean-meal				*Soya*		
5	307·5	23·9	6·9	3·4	27·8	10·4	38·2	85
10	1297·5	100·9	28·9	14·4	117·3	43·8	161·1	263
15	2647·5	205·9	58·9	29·4	239·3	89·4	328·7	580
20	4717·5	366·9	104·9	52·4	426·5	159·2	585·7	1014
25	7192·5	559·4	159·9	79·9	650·2	242·8	893·0	1426
		Dried alfalfa				*Alfalfa*		
5	151·1	48·8	4·2	2·1	13·7	6·0	19·7	35
10	543·7	183·0	15·1	7·6	49·1	22·7	71·8	141
15	1451·9	493·2	40·3	20·1	131·2	61·1	192·3	323
20	2753·3	937·8	76·3	38·1	248·9	116·1	365·0	600
25	4633·1	1580·0	128·3	64·1	418·8	195·6	614·4	1145
		Dried grass				*Grass*		
5	154·2	52·7	4·3	2·1	13·9	6·5	20·4	35
10	629·2	215·0	17·3	8·7	56·9	26·6	83·5	159
15	1537·3	525·2	42·5	21·3	139·0	65·0	204·0	346
20	2983·3	1091·1	82·3	41·2	269·7	126·2	395·9	622
25	4911·3	1677·8	135·6	67·8	444·0	207·7	651·7	1063

Table II (*cont.*).

D. *Series 2.*

Figures represent g.

In weeks	Wheat	Fish-meal	Cod-liver oil	Salts	Protein: Wheat	Protein: *Fish*	Protein: Total	Weight increase
5	361·8	31·7	4·1	2·8	32·7	18·2	50·9	121
10	2101·8	183·7	23·3	16·3	190·0	105·6	295·6	506
15	5491·8	479·8	60·8	42·4	496·5	275·9	772·4	1145
20	9220·8	805·5	102·1	71·1	833·6	463·2	1296·8	1780
		Meat-meal				*Meat*		
5	343·4	25·5	3·8	3·8	31·0	15·3	46·3	86
10	1099·5	81·9	12·1	12·1	99·4	49·1	148·5	277
15	2680·3	199·7	29·5	29·5	242·3	119·6	361·9	579
20	4732·3	352·7	52·0	52·0	427·8	211·3	639·1	1073
25	8152·3	607·7	89·5	89·5	737·0	364·0	1101·0	1839
		Fat-free meat-meal				*Meat*		
5	460·9	25·4	5·0	7·9	41·7	22·8	64·5	132
10	1937·6	106·9	21·1	33·3	175·2	95·8	271·0	452
15	4591·1	253·4	49·9	79·3	415·0	227·0	642·0	1046
20	7706·1	425·4	83·7	133·3	696·6	381·2	1077·8	1630
		Blood-meal				*Blood*		
5	314·5	17·4	3·3	3·3	28·4	13·9	42·3	98
10	1262·1	69·4	13·5	13·5	114·1	55·4	169·5	283
15	2822·7	155·2	30·3	30·3	255·2	123·8	379·0	577
20	4717·8	259·3	50·7	50·7	426·5	206·9	633·4	900
25	8062·3	442·8	86·7	86·7	728·8	353·3	1082·1	1484
		Caseinogen				*Caseinogen*		
5	347·8	22·5	7·6	3·9	31·4	17·4	48·8	107
10	1268·0	82·1	27·8	14·0	114·6	63·6	178·2	359
15	3363·3	217·8	73·8	37·0	304·0	168·8	472·8	915
20	6369·6	412·5	139·8	70·0	575·8	319·7	895·6	1532
		Lact-albumin				*Lact-albumin*		
5	267·0	24·0	6·0	3·0	24·1	13·5	37·6	63
10	909·7	82·4	20·5	10·2	82·2	46·5	128·7	200
15	2469·1	224·3	55·6	27·6	223·2	126·5	349·7	513
20	4862·6	442·3	109·4	54·6	439·6	249·5	689·1	1056
		Dried egg-white				*Egg-white*		
5	247·6	16·5	5·4	2·7	22·4	12·3	34·7	64
10	834·9	55·9	18·3	9·1	75·5	41·7	117·2	192
15	2149·4	144·1	47·2	23·6	194·3	107·5	301·8	522
20	4467·7	299·5	98·2	48·9	403·5	223·4	626·9	1000
25	7442·6	499·6	163·8	81·6	672·8	372·7	1045·5	1479
		Dried egg-yolk				*Egg-yolk*		
5	321·8	19·9	7·0	3·6	29·1	15·4	44·5	92
10	1232·8	77·5	27·0	13·6	111·4	60·1	171·5	338
15	2933·3	187·6	64·3	32·3	265·2	145·4	410·6	761
20	5544·8	356·7	121·6	61·0	501·3	276·4	777·7	1336
		Fresh egg-white				*Egg-white*		
5	683·5	435·1	11·3	11·3	61·8	46·6	108·4	283
10	2460·1	1563·0	40·9	40·9	222·4	167·2	389·6	920
15	5490·2	3489·5	91·3	91·3	496·3	373·4	869·7	1734
20	9087·6	5771·6	151·3	151·3	821·5	617·6	1439·1	2285

Table II. D. *Series 2 (cont.)*.

In weeks	Wheat	Fresh egg-yolk	Cod-liver oil	Salts	Protein: Wheat	Protein: *Egg-yolk*	Protein: Total	Weight increase
5	520·9	190·9	7·3	7·3	47·1	29·6	76·7	167
10	1556·8	570·7	21·8	21·8	140·7	88·5	229·2	458
15	3085·9	1132·1	43·3	43·3	279·0	175·5	454·5	998
20	4893·4	1795·0	68·7	68·7	442·4	278·2	720·6	1242
		Dried yeast (*a*)				*Yeast*		
5	439·1	48·1	9·4	10·2	39·7	23·0	62·7	116
10	1419·5	155·7	32·0	32·8	128·3	74·4	202·7	325
15	2572·9	282·3	58·6	59·4	232·6	135·0	367·6	616
20	4533·5	497·7	104·0	104·8	409·8	237·9	647·7	1079
		(*b*)						
5	513·7	55·6	5·8	5·8	46·4	26·6	73·0	125
10	1987·6	214·9	22·6	22·6	179·7	102·7	282·4	474
15	4198·6	453·9	47·6	47·6	379·5	217·0	596·5	878
20	6970·7	766·5	107·4	79·0	630·1	361·6	991·7	1338
		Dried germ (*a*)				*Wheat germ*		
5	347·8	64·4	8·5	8·5	31·4	19·8	51·2	121
10	1562·8	289·4	38·5	38·5	141·3	88·8	230·1	455
15	3668·8	679·4	90·5	90·5	331·7	208·6	540·3	944
20	6422·8	1189·4	158·5	158·5	580·6	365·1	945·7	1601
		(*b*)						
5	320·5	58·3	3·9	6·1	29·0	17·9	46·9	81
10	1486·6	270·8	18·2	28·7	134·4	83·1	217·5	384
15	3574·1	650·8	43·5	69·2	323·1	199·8	522·9	913
20	6952·5	1265·8	84·5	134·8	628·5	388·6	1017·1	1621
		Split peas				*Peas*		
5	201·4	64·4	5·4	2·7	18·2	12·5	30·7	74
10	747·1	238·1	20·2	10·0	67·5	46·4	113·9	217
15	1645·3	525·2	44·4	22·1	148·7	102·3	251·0	444
20	2952·0	942·8	79·6	39·7	266·9	183·7	450·6	780
25	4942·6	1579·3	133·7	66·8	446·8	307·7	754·5	1292
		Split lentils				*Lentils*		
5	184·7	48·3	4·8	2·4	16·7	11·0	27·7	47
10	692·1	181·0	17·8	8·9	62·6	41·1	103·7	125
15	1358·5	355·2	35·0	17·5	122·8	80·7	203·5	232
20	1973·6	516·0	50·8	25·4	178·4	117·2	295·6	335
25	2742·6	717·0	70·7	35·3	247·9	162·8	410·7	444
		Bean-meal				*Bean*		
5	174·0	43·7	4·5	2·3	15·7	10·5	26·2	43
10	950·5	260·2	25·0	12·5	85·9	62·4	148·3	227
15	2424·6	726·3	65·1	32·4	219·2	174·2	393·4	476
20	3898·7	1192·4	105·2	52·3	352·4	286·1	638·5	805
25	5864·0	1813·7	158·5	79·0	530·1	435·1	965·2	1156
		Soya bean-meal				*Soya*		
5	275·0	33·4	6·4	3·2	24·9	14·5	39·4	70
10	1284·5	155·9	29·8	14·8	116·1	67·7	183·8	286
15	2841·5	344·9	65·8	32·8	256·9	149·7	406·6	641
20	5263·5	638·9	121·8	60·8	475·8	277·3	753·1	1197
25	8550·5	1037·9	197·8	98·8	773·0	450·5	1223·5	1859

Table II. D. *Series 2 (cont.)*.

In weeks	Wheat	Dried alfalfa	Cod-liver oil	Salts	Protein: Wheat	Protein: *Alfalfa*	Protein: Total	Weight increase
5	161·5	99·6	5·4	2·7	14·6	12·3	26·9	41
10	611·5	377·1	20·4	10·2	55·3	46·7	102·0	142
15	1291·8	796·7	43·2	21·7	116·8	98·6	215·4	298
20	2234·6	1378·0	74·5	37·6	202·0	170·6	372·6	505
25	3605·9	2223·8	120·0	60·4	326·0	275·3	601·3	821
		Dried grass				*Grass*		
5	136·5	84·3	4·6	2·3	12·3	10·4	22·7	35
10	633·7	390·9	21·2	10·5	57·3	48·4	105·7	111
15	1353·7	834·9	45·2	22·5	122·4	103·4	225·8	230
20	2253·7	1389·8	75·2	37·6	203·7	172·1	375·8	428
25	3403·7	2098·9	113·4	56·7	307·7	259·8	567·5	689
		Gluten						
5	153·7	10·5	1·7	1·7	13·9	7·7	21·6	18
10	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—

E. *Series 3*.

Figures represent g.

In weeks	Wheat	Fish-meal	Cod-liver oil	Salts	Protein: Wheat	Protein: *Fish*	Protein: Total	Weight increase
5	514·7	64·4	5·9	—	46·5	37·0	83·5	169
10	2114·7	264·4	24·1	—	191·2	152·0	343·2	736
15	4994·7	624·4	56·8	—	451·5	359·0	810·5	1498
20	8642·7	1080·4	98·2	—	781·3	621·1	1402·5	2288
		Meat-meal				*Meat*		
5	415·5	48·3	4·7	4·7	37·6	28·9	66·5	105
10	1679·8	195·2	19·1	19·1	151·9	116·9	268·8	454
15	3874·8	450·2	44·1	44·1	350·3	269·7	620·0	949
20	7035·6	817·4	80·1	80·1	636·0	489·6	1125·6	1603
		Fat-free meat-meal				*Meat*		
5	373·5	28·1	8·7	4·1	33·8	25·2	59·0	123
10	1815·5	136·1	42·0	19·8	164·1	121·9	286·0	471
15	4067·7	306·7	94·2	44·7	367·7	275·7	643·4	1006
20	6845·9	516·3	158·4	75·4	618·9	463·5	1082·4	1534
		Blood-meal				*Blood*		
5	437·5	36·8	4·8	4·8	39·6	29·4	69·0	91
10	1801·6	152·1	27·0	20·0	162·9	121·3	284·2	302
15	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—
		Caseinogen				*Caseinogen*		
		(*a*)						
5	309·4	27·4	6·9	3·4	27·9	21·2	49·1	110
10	1434·7	126·7	32·2	16·0	129·7	98·2	227·9	320
15	3514·4	310·3	78·7	39·3	317·7	240·5	558·2	796
20	6188·3	546·5	138·6	69·4	559·4	423·5	982·9	1291
		(*b*)						
5	231·9	20·8	10·7	2·7	21·0	16·1	37·1	75
10	1218·1	108·5	45·5	13·9	110·1	84·1	194·2	344
15	3049·3	272·1	129·5	34·9	275·7	210·9	486·6	906
20	5229·3	467·1	229·5	59·9	472·7	362·0	834·7	1296

Table II. E. *Series 3 (cont.).*

In weeks	Wheat	Lact-albumin	Cod-liver oil	Salts	Protein: Wheat	Protein: *Lactalbumin*	Protein: Total	Weight increase
5	329·4	41·3	7·7	3·8	29·8	23·3	53·1	90
10	1366·2	171·2	31·7	15·9	123·5	96·6	220·1	293
15	2982·6	373·7	69·2	34·6	269·6	210·8	480·4	700
20	5245·5	657·2	121·7	60·8	474·2	370·7	844·9	1200
		Fresh egg-white				*Egg-white*		
5	345·7	480·7	8·4	8·4	31·3	51·4	82·7	198
10	1825·3	2537·5	44·4	44·4	165·0	271·5	436·5	850
15	4113·2	5721·7	100·3	100·3	371·8	612·2	984·0	1626
20	6663·1	9272·6	162·6	162·6	602·3	992·2	1594·5	1930
		Fresh egg-yolk				*Egg-yolk*		
5	232·5	156·3	4·0	4·0	21·0	24·2	45·2	99
10	1133·3	761·8	19·2	19·2	102·5	118·1	220·6	448
15	2740·3	1842·2	46·6	46·6	247·7	285·5	533·2	906
20	4465·6	3002·0	76·0	76·0	403·7	465·3	869·0	1433
		Dried yeast				*Yeast*		
5	376·9	56·9	9·1	9·1	34·1	27·2	61·3	123
10	1353·9	204·5	32·5	32·5	122·4	97·8	220·2	384
15	3272·1	494·3	78·5	78·5	295·8	236·3	532·1	795
20	5774·1	872·3	138·5	138·5	522·0	417·0	939·0	1268
		Dried wheat germ (*a*)				*Wheat germ*		
5	683·3	177·5	8·8	17·7	61·8	54·5	116·3	316
10	2685·3	697·5	34·8	69·7	242·8	214·1	456·9	1015
15	5765·3	1497·5	74·8	149·7	521·2	459·7	980·9	1891
20	8229·3	2137·5	106·8	213·7	743·9	656·2	1400·1	2304
		(*b*)						
5	405·3	106·7	10·7	10·7	36·6	32·8	69·4	140
10	1469·3	386·7	38·7	38·7	132·8	118·7	251·5	496
15	3217·3	846·7	84·7	84·7	290·8	259·9	550·7	1040
20	5877·3	1546·7	154·7	154·7	531·3	474·8	1006·1	1675
		(*c*)						
5	367·6	95·6	10·1	4·7	33·2	29·3	62·5	151
10	1778·2	469·9	49·4	23·4	160·7	144·3	305·0	641
15	4250·1	1112·8	116·9	55·5	384·2	341·6	725·8	1340
20	7216·2	1884·2	197·9	94·1	652·2	578·5	1230·7	1988
		Gluten				*Gluten*		
5	392·0	37·6	9·1	4·2	35·4	27·7	63·1	86
10	1524·8	146·4	34·7	17·0	137·8	107·9	245·7	292
15	3224·0	309·6	73·1	36·2	291·5	228·2	519·7	627
20	5631·2	540·8	127·5	63·4	509·0	398·6	907·6	1013

Consideration of the results.

Osborne *et al.* [1919] pointed out that the nutritive values of proteins could be expressed numerically when the animals ate the same amount of food of the same caloric value in the same number of days and gained the same weight, the amount and kind of protein in the diet being the only variable. It was recognised that it was exceedingly difficult to conduct such experiments, and on account of individual variations large numbers of animals were necessary.

Many of our data conform to these conditions especially where the values of the proteins are close together, such as meat and fish, yeast and wheat germ,

grass and alfalfa, but there is no agreement where the values of the proteins are widely different, such as caseinogen and lentils or grass. Comparisons cannot be made by this method in all cases.

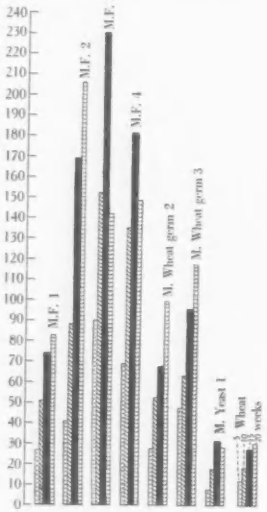

Fig. 1. *Maize series.*

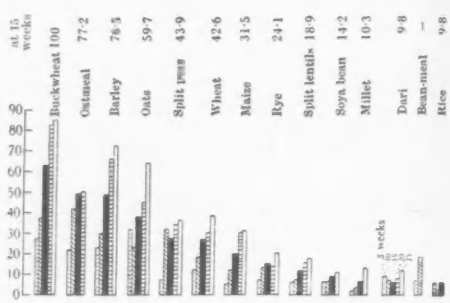

Fig. 2. *Series 0.*

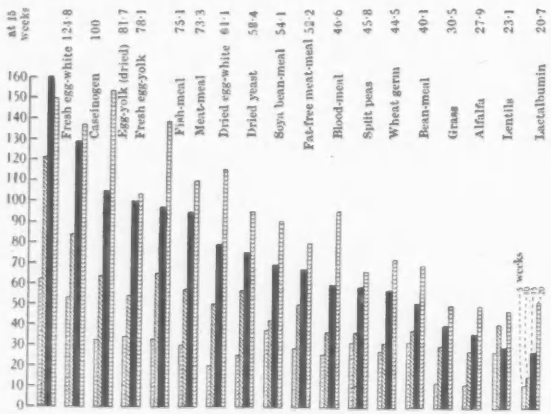

Fig. 3. *Series 1.*

Our diets are of approximately equal caloric value and contain almost the same amount of total protein with exactly the same amount of added protein. The best protein will give the greatest increase with the smallest consumption of protein in the shortest time. Increase I, time T, and protein consumption P

together represent the value of the protein in nutrition, *i.e.* I/T is the first factor and I/P is the second factor. The product $I/T \times I/P$ or I^2/TP, represents the value of the protein numerically.

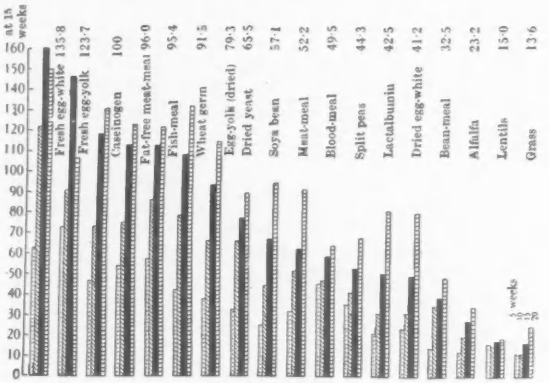

Fig. 4. *Series 2.*

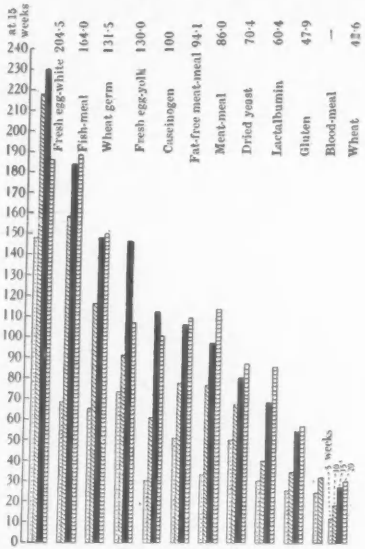

Fig. 5. *Series 3.*

I/T is the growth curve. We have constructed the growth curves of the cocks and hens in each series of experiments. They are of great interest and show in a large measure the different values of the proteins, but they are not included in this publication.

We have calculated the values of I^2/TP, taking 5-week periods for convenience. Any other period would serve equally well. The figures are given in

Table III A to E. The values could also be calculated by fixing the increase at 250, or 500 or 1000 g., noting the time and protein consumption to reach the amount of the increase. A series of charts (Figs. 1–5), showing the figures of Table III, has been drawn in which the 5-week periods are indicated by different shadings.

The charts (and also the growth curves) often show that the increase may be small in the first 5 weeks, probably owing to the day-old chicks not being equally vigorous and not liking the diet, with more rapid growth during the next 5 weeks, thus catching up the poor start of the earlier period. By 15 weeks the birds have become accustomed to the food and are growing steadily. On good diets, after 15 weeks the rate of growth often declined. The birds were becoming sexually mature, and in a few experiments the hens started laying about the 20th week. For final comparison we are inclined to take the figures at 15 weeks, except in the series 0 on cereals alone in which growth was slower; the figures at 20 weeks are preferable here.

If we exclude the figures for fresh egg-white and fresh egg-yolk, which on the protein ratio (Table I) may equally well belong to the next series, caseinogen, except in series 3, is the best protein. By fixing its value at 100 and calculating the other comparative values on this basis, a set of relative values is obtained. These are also given in Table III A to E and show more clearly the differences in the proteins. The relative values at 15 weeks are shown on the charts.

Table III.

A. *Series 0.*

	I^2/TP Weeks					Relative values Weeks				
	5	10	15	20	25	5	10	15	20	25
Buckwheat	29·5	41·6	64·0	84·3	95·2					
	24·0	33·1	62·2	80·6	75·1					
Mean	26·8	37·4	63·1	82·5	85·2	100	100	100	100	100
Barley (1)	17·1	32·9	55·8	79·2	89·3					
(2)	29·1	27·1	40·8	53·3	65·1					
Mean	23·1	30·0	48·3	66·3	72·2	86·2	80·2	76·5	80·4	84·7
Oatmeal (1)	23·3	46·6	60·6	59·7	—					
(2)	18·2	37·5	36·7	39·8	33·1					
Mean	20·8	42·1	48·7	49·8	—	77·6	112·6	77·2	60·4	—
Oats	31·8	23·6	37·7	45·0	63·7	118·6	63·1	59·7	54·5	74·8
Split peas	7·5	31·9	27·7	34·6	36·6	28·0	85·3	43·9	41·9	43·0
Wheat (1)	15·3	22·0	31·2	39·9	53·4					
(2)	8·9	14·7	22·6	20·6	23·3					
Mean	12·1	18·4	26·9	30·3	38·4	45·1	49·2	42·6	36·7	45·1
Maize (1)	5·0	11·9	19·9	30·3	31·3	18·6	31·8	31·5	36·7	36·7
(2)	1·0	4·1	—	—	—					
Rye (1)	10·7	17·8	16·3	15·3	27·3					
(2)	3·7	8·9	14·0	12·8	14·8					
Mean	7·2	13·4	15·2	14·1	21·0	26·9	35·8	24·1	17·1	24·6
Split lentils	6·0	6·7	11·9	15·6	17·9	22·4	18·0	18·9	18·9	21·0
Soya bean-meal	6·5	6·6	9·0	11·1	—	24·2	17·6	14·2	13·4	—
Millet (1)	1·9	3·6	6·5	10·9	—	7·1	9·4	10·3	13·2	—
(2)	2·0	3·4	3·7	—	—					
Dari (1)	9·1	7·5	6·2	8·0	12·0	34·0	20·1	9·8	9·7	14·1
(2)	2·2	2·3	2·8	—	—					
Brown rice (1)	3·2	7·8	—	—	—					
(2)	6·0	2·5	6·2	—	—	22·4	6·9	9·8	—	—
Bean-meal	7·1	18·6	—	—	—	26·5	49·5	—	—	—

Table III (*cont.*).

B. *Maize series.*

	Protein ratio	I^2/TP Weeks 5	10	15	20	25
Fish-meal (1)	1 : 2·5	26·4	51·2	73·7	83·2	82·1
(2)	1 : 1·2	40·7	88·2	169·0	206·2	210·5
(3)	1 : 0·75	90·4	152·4	230·0	141·9	—
(4)	1 : 0·52	68·7	135·0	181·1	148·2	—
Dried yeast (1)	1 : 2·4	7·7	17·6	31·2	28·0	26·0
Wheat germ (2)	1 : 1·04	27·3	52·5	69·7	99·8	—
(3)	1 : 0·6	47·3	62·8	95·3	117·8	—

C. *Series 1.*

	I^2/TP Weeks 5	10	15	20	Relative values Weeks 5	10	15	20
Caseinogen	53·5	84·1	128·7	137·5	100	100	100	100
Egg-white	62·4	121·7	160·6	149·5	116·6	144·7	124·8	108·7
Dried egg-yolk	32·8	64·0	105·2	154·0	61·2	76·1	81·7	112·0
Fresh egg-yolk	34·5	54·5	100·5	102·9	64·5	64·8	78·1	74·9
Fish-meal	33·2	65·4	97·6	138·9	62·0	77·7	75·8	101·0
Meat-meal	30·0	57·4	94·4	109·9	56·1	68·3	73·3	79·9
Dried egg-white	19·8	48·8	78·7	114·8	35·1	58·0	61·1	83·5
Dried yeast	24·5	56·7	75·2	95·3	45·8	67·4	58·4	69·3
Soya bean-meal	37·4	42·1	69·6	91·3	70·0	50·1	54·1	66·4
Fat-free meat-meal	28·9	50·2	67·2	79·7	54·0	59·7	52·2	58·0
Blood-meal	25·9	36·3	60·0	96·2	48·4	43·2	46·6	70·0
Split peas	31·9	36·7	58·9	66·3	59·6	43·6	45·8	48·2
Dried wheat germ	27·5	31·9	57·3	72·3	51·4	37·9	44·5	52·6
Bean-meal	32·2	37·4	51·6	69·1	60·2	44·5	40·1	50·3
Dried grass	12·0	30·2	39·3	48·8	22·4	35·9	30·5	35·5
Dried alfalfa	11·0	27·6	35·9	49·2	20·5	32·8	27·9	35·8
Split lentils	27·0	40·2	29·8	47·2	50·5	47·8	23·1	34·4
Lactalbumin	11·2	15·7	26·7	51·7	20·9	18·7	20·7	37·6

D. *Series 2.*

	I^2/TP Weeks 5	10	15	20	Relative values Weeks 5	10	15	20
Caseinogen	46·9	73·6	118·3	131·0	100	100	100	100
Fresh egg-white	62·4	121·7	160·6	149·5	133·0	165·3	135·8	114·1
Fresh egg-yolk	72·8	91·6	146·3	106·8	155·2	124·3	123·7	81·5
Fat-free meat-meal	54·1	75·5	113·6	123·1	115·3	102·5	96·0	94·0
Fish-meal	57·6	86·5	112·9	121·9	122·8	117·5	95·4	93·0
Wheat germ (*a*)	57·1	90·1	110·1	135·2	121·7	122·4	93·1	103·2
(*b*)	28·0	67·6	106·6	129·6	59·7	91·8	90·1	98·9
Mean	42·6	78·9	108·3	132·4	90·8	107·1	91·5	101·1
Dried egg-yolk	38·1	66·2	93·8	114·9	81·2	89·9	79·3	87·7
Dried yeast (*a*)	23·1	52·0	69·0	90·2	49·2	70·6	58·3	68·9
(*b*)	42·7	79·6	86·0	90·3	91·0	108·1	72·7	68·9
Mean	32·9	65·8	77·5	90·2	70·1	89·4	65·5	68·9
Soya bean-meal	24·9	44·3	67·5	95·2	53·1	60·2	57·1	72·7
Meat-meal	32·0	51·8	61·8	90·2	68·2	70·4	52·2	39·9
Blood-meal	45·5	47·3	58·5	63·9	97·0	64·3	49·5	48·8
Split peas	35·7	41·2	52·4	67·5	76·1	56·0	44·3	51·5
Lactalbumin	21·2	31·0	50·3	80·8	45·2	42·1	42·5	61·7
Dried egg-white	23·5	31·5	48·7	80·0	50·1	42·8	41·2	61·1
Bean-meal	14·1	34·7	38·4	50·6	30·1	47·1	32·5	38·6
Alfalfa	12·5	19·7	27·5	34·2	26·6	26·8	23·2	26·1
Split lentils	16·0	15·1	17·7	19·0	34·1	20·5	15·0	14·5
Dried grass	11·4	11·7	16·1	24·4	24·3	15·9	13·6	18·6

Table III (*cont.*).

E. *Series 3.*

	I^2/TP Weeks				Relative values Weeks			
	5	10	15	20	5	10	15	20
Caseinogen (1)	49·2	45·2	75·7	84·8				
(2)	30·3	60·9	112·5	100·6	100	100	100	100
Fresh egg-white	147·7	217·1	230·0	186·3	487·4	356·5	204·5	185·2
Fish-meal	68·4	157·8	184·5	186·6	225·7	259·1	164·0	185·5
Wheat germ (1)	171·7	225·5	243·0	199·5				
(2)	56·5	97·8	130·9	139·4				
(3)	72·9	134·3	164·9	160·5				
Mean of (1) and (2)	64·7	116·0	147·9	150·0	213·5	190·5	131·5	144·1
Fresh egg-yolk	72·8	91·6	146·3	106·8	240·3	150·4	130·0	106·1
Fat-free meat-meal	51·3	77·5	105·9	108·7	169·3	127·2	94·1	108·0
Meat-meal	33·2	76·3	96·8	113·7	109·6	125·3	86·0	113·0
Dried yeast	49·3	67·0	79·2	85·6	162·7	110·0	70·4	85·1
Lactalbumin	30·5	39·0	68·0	85·2	100·6	64·0	60·4	84·7
Gluten	23·4	34·7	53·9	56·5	77·2	57·0	47·9	56·1
Blood-meal	24·0	32·0	—	—	79·2	52·5	—	—

Series 0. The most interesting and clearest results are with cereals and pulses alone. The best growth and the highest comparative value were shown by buckwheat. The chickens also looked very healthy. It was followed by oatmeal and barley, the latter being superior at 20 weeks. The birds also looked healthy on barley, not so well on oatmeal. Wheat had about half the value of buckwheat. The difference in the two experiments may be due to the poorer quality of the wheat in the second trial. Next in order is maize from the values in one experiment only; in this trial the result may be fictitious as birds died during the night and were pecked by the others who thus obtained some additional protein.

The close similarity of wheat and maize was also observed by Boas-Fixsen *et al.* [1934]. If their data be calculated on our basis they come out as 7·4 for wheat and 6·7 for maize. On millet, dari, brown rice and bean-meal the chicks did not survive 25 weeks. The proteins of these seeds must be considered as very poor. The protein of rice, often considered of high value, is of no value for chickens.

Amongst the pulses, split peas were superior to soya bean, which was inferior to expectation. The value of soya bean protein was higher in combination with wheat. The inferior values of the pulses are evidently due to the small amount of cystine in their proteins as noted by other workers.

Maize series. The maize series was carried out primarily to ascertain the effect of increasing quantities of fish-meal in the diet for the purpose of finding out at what "level of protein intake" the trials should be made. The charts give a very good picture of the results. They show clearly that the best quantity of fish-meal is 15 % (MF_3). The growth was distinctly less with 20 % of fish-meal. With wheat germ corresponding to 10 and 15 % of fish-meal the growth and comparative value were distinctly less, about half that of the fish-meal. Similarly, with yeast corresponding to 5 % of fish-meal the comparative figure was about half. These trials were made also to find out the effect of extra vitamin B_1 in the diet. The growth did not become greater, as might have been expected, and actually depends upon the nature of the protein.

Wheat series. The data of relative values which might have been expected to come out in the same order on the three series of diets are unfortunately erratic and it is not possible to give the explanation. Several suggestions may be offered. The first is that the health of the chicks at the start was not uniform. Most of the birds of each series died generally during the first few weeks; *post mortem* examination did not show any special reason. We are inclined to suspect that the wheat supplied too little vitamin B_1 which effect would be most noticeable at the start. We found previously that to rear chicks on oatmeal it was necessary to add some marmite. The second is that the difference in numbers of the sexes in the various trials would lead to variations in the food consumption for which no allowance can be made. The sexes of the birds are not known for some weeks and in the case of the poor proteins often not till *post mortem* examination.

Palatability of the food is another suggestion. It was obvious that blood-meal, lactalbumin, grass and alfalfa were not liked by the chicks and were left over after the wheat had been consumed. A further possibility is in the reckoning of the consumption; if a bird died during a week it was difficult to judge whether it had or had not consumed food. Some food may have been lost by scattering or have been blown away if the weather was very windy.

In series 3 the low value of caseinogen is remarkable and further there was high mortality in both experiments, not avoided by the extra cod-liver oil. From our previous experience on the vitamin B_1 needs and the necessity of balance of food by vitamin B_1 we think that with more rapid growth produced by more animal protein more vitamin B_1 is wanted. The mortality was not so high with wheat germ. Omitting the caseinogen results the other values come out in much the same order as in series 1 and 2.

On comparing series 1 and 2 it is noticeable that wheat germ gives a higher relative value in series 2. If it were due to the effect of extra vitamin B_1, the same higher value should have been given with yeast. Dried egg-white in series 2 is too low in the scale. The larger quantity may have been unpalatable, it was not observed by us to have been scattered and wasted. The same reasons may apply to the low value of meat-meal in series 2 and fat-free meat-meal in series 1. The two should come out closer.

If one takes the mean values of the relative numbers of series 1 and 2, one can arrange the proteins in an order which is probably correct except in one or two cases. The order is:

Fresh egg-white	130·6	Soya bean	55·6
Fresh egg-yolk	100·9	Dried egg-white	51·2
Caseinogen	100·0	Blood-meal	48·0
Fish-meal	85·3	Split peas	45·0
Dried egg-yolk	80·5	Bean-meal	36·3
Fat-free meat-meal	74·1	Lactalbumin	31·6
Wheat germ	68·0	Alfalfa	25·6
Meat-meal	62·8	Grass	22·0
Dried yeast	62·0	Lentils	19·0

Dried egg-white is obviously too low, and the figure must be looked upon as erroneous. Meat-meal is too low and should have the same value as fat-free meat-meal. The blood-meal value seems low but may be explained from its protein value being determined from the nitrogen content, some of which is from haemoglobin. The lactalbumin figure is too low, due, as explained previously, to the poor sample. In general, the results are in accord with those of previous workers that animal are superior to vegetable proteins.

We desire to express our thanks for grants from the Government Grant Committee of the Royal Society and from Imperial Chemical Industries, which have covered the expenses of these experiments.

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CCXLVI. THE EFFECTS OF INOSITOL, CRYSTALLINE VITAMIN B_1 AND "PANTOTHENIC ACID" ON THE GROWTH OF DIFFERENT STRAINS OF YEAST.

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That inositol is a constituent of Wildiers' "bios" and hence is involved in yeast-growth stimulation, was first suggested by Eastcott [1928], but some doubt was entertained regarding her findings by Williams, R. J., Warner and Roehm [1929] and Narayanan [1930].

The possible effect of "vitamin B" on yeast growth was suggested by Williams, R. J. [1919], and later evidence was presented [Williams, R. J. and Roehm, 1930] that the antineuritic factor is one substance involved in the growth stimulation of certain yeasts. The crystalline hydrochloride of vitamin B_1 (recrystallised from alcohol) has recently become available to us through the courtesy of R. R. Williams [Williams, R. R. *et al.*, 1934]. While conclusive evidence as to the purity of this preparation has not been published, it is effective in very small doses (5γ are a curative dose for rats), and there is no reason at this time to doubt its essential purity, particularly from the physiological standpoint.

The term "pantothenic acid" (from Greek meaning "from everywhere") has been applied to what is believed to be a single acid substance of universal biological occurrence [Williams, R. J. *et al.*, 1933]. This acid (or possibly acids) has a striking effect on the growth of a particular strain of yeast ("Gebrüder Mayer") and its presence is tested for quantitatively by this effect. The "active principle" from whatever source is always an acid (or mixture of acids) of about the same strength. Using rice bran as a source, the ionisation constant is determined by fractional electrolysis to be about 3×10^{-5} [Williams, R. J. and Moser, 1934]. Regardless of the source, the active substance behaves in a variety of ways as essentially the same substance; it is not destroyed by catalytic hydrogenation with or without a promoter; it is not destroyed by oxidation with ammoniacal silver solution; its heat-stability at different hydrogen-ion concentrations is consistent; it is destroyed by nitrous acid; it is capable of esterification with alcohol; the esters are inactive physiologically but the activity returns on hydrolysis. The esters are more soluble in water than in ether. Diffusion studies indicate that the molecular weight (of material from different sources) is consistently of the order of 150–200.

Based upon this information we have worked out in this laboratory a method for concentrating the acid, using autolysed liver as a source, and have succeeded in obtaining it of such potency that about 2·5 parts per 10^{10} parts of culture medium can be detected by its effect on yeast growth. This is equivalent to 1 mg. per 4000 litres of culture medium. In all the numerous fractionations we have performed we have never had reason to suspect that the "active principle"

is more than one substance. Although inherently difficult to purify, it always has a tendency to become concentrated at one point in a fractionation procedure rather than to distribute itself widely as might be the case if two or more substances were involved. We constantly make use of the fact that it is an acid, and the solubilities of its salts are those that would be expected of a polyhydroxy-acid of relatively low molecular weight as previously conceived [Williams, R. J. *et al.*, 1933].

The methods of purification are undergoing active investigation and the details of procedure will be published soon. The preparation used in this study is about one-fourth as potent as the most active preparation yet made in this laboratory. While it is obviously not a pure chemical substance, we have no reason to think that it contains any substance which affects yeast growth, other than the acid which is described above.

In testing the effects of the three materials mentioned above—inositol, crystalline vitamin B_1, and "pantothenic acid"—on various yeasts we obtained results much more striking than was anticipated, and since they go so far toward clearing up the "bios question," we decided to offer them for publication without waiting for final purification of the agent which has been concentrated in this laboratory.

The strains of *Saccharomyces cerevisiae* chosen for study include those used by various workers who have attempted to determine the nature of the yeast growth stimulants. Included are Wildiers' yeast (obtained through the courtesy of Dr M. Ide), "old process" yeast (used by the senior author in 1919), W. L. Miller's yeast obtained from Toronto, Yeast #2190 obtained through the courtesy of Dr J. C. Drummond, "Gebrüder Mayer" yeast obtained from the Fleischmann Co. and used by W. H. Eddy in his studies, and yeast taken directly from a fresh Fleischmann cake. All of these with the exception of the last have been grown in stock culture for years on molasses agar, made up from the same batch of blackstrap molasses [Williams, R. J. *et al.*, 1927].

The medium which has been used in these studies for several years has been modified to meet the suggestions of Elvehjem [1931] regarding the stimulating effect of iron and copper, of Richards [1932] regarding a similar effect of thallium, as well as the recognised facts that traces of boron are required by many plants and that traces of zinc, manganese and iodine are needed by at least some plant organisms. Each litre of the modified medium, therefore, contains the following solid ingredients: sucrose 20 g.; $(NH_4)_2SO_4$, 3·0 g.; KH_2PO_4, 2·0 g.; asparagine, 1·5 g.; $CaCl_2, 2H_2O$, 0·25 g.; $MgSO_4$, 0·25 g.; $TlCl_3$, 1·0 mg.; $ZnSO_4$, 1·0 mg.; $MnCl_2$, 1·0 mg.; H_3BO_3, 1·0 mg.; $FeCl_3$, 0·5 mg.; $CuSO_4$, 0·1 mg.; KI, 0·1 mg.

It should be remarked in this connection that we regard the presence of asparagine or some substitute therefor to be necessary. In unpublished experiments conducted by Truesdail it was demonstrated that asparagine (recrystallised many times) may become a definite limiting factor for the growth of "Gebrüder Mayer" yeast when plenty of nutrilite (growth factor) is present. According to previous experience, this was not likely to be the case with "old process" yeast. We suspect that the effects of extracts used by other workers when added to an asparagine-free medium, may be due in part to organic nitrogen, unless the yeasts are quite different in their responses from "Gebrüder Mayer" yeast.

We have recognised for some time that traces of inorganic materials such as those added might have a stimulating effect on yeast growth. We noted in one experiment [Williams, R. J. and Truesdail, 1931] the slight improvement brought about by yeast ash. Such effects, however, are small (from the stand-

point of our usual growth responses) and in part at least due to the presence of traces of these substances as impurities in our salts. The addition of the traces of salts indicated to our medium has a detectable favourable effect on yeast growth, but the effect is too small to have had a significant effect on our previous results. We believe, however, that it is safer to introduce the traces of all of these salts knowingly, rather than to rely on chance.

The number of combinations (quantitative as well as qualitative) of the three factors we are studying might be extended almost indefinitely, hence we have had to make distinct limitations. The effect of inositol in different concentrations when used in the presence of the other factors in different amounts has not been studied because in the case of the "Gebrüder Mayer" yeast previously studied in this connection variation of the concentrations of inositol in the general range of 1–10γ per ml. made little difference in the response obtained in our tests as ordinarily carried out. A dosage of about 20γ per ml. is used under the conditions of the tests as carried out in the Toronto laboratories [Miller *et al.*, 1933].

Since previous publications from this laboratory, we have made an improvement in our method of seeding. The unknown materials to be tested are made up to 2 ml. with water, sterilised and then seeded with 10 ml. of a very dilute suspension of yeast in control medium. This suspension is made by dilution from a heavier suspension which is standardised by means of our thermocouple apparatus [Williams, R. J., *et al.*, 1929]. We find that this procedure allows for a more constant seeding and more uniform responses.

For all the yeasts except the last two, the seeding was 0·12 mg. moist yeast per culture. For the two others which are more rapidly growing yeasts, the seeding was 0·04 mg. The growth period was 18 hours at 30° and the crops are given in terms of moist yeast per ml. Each series of 27 cultures was run upon a separate day. The duplicate tests on the same yeasts were in most cases separated by a few weeks, and the differences between the magnitudes of the growths are no doubt due to differences in exact age and history of the particular cultures used. The relative effects of the different additions are in general concordant. The seeding from the Fleischmann cake was made directly from the centre of a cake without first isolating a pure culture. That the growth is somewhat irregular is possibly due to this cause.

Our experience agrees with that of Miller *et al.* [1933] in that, especially when growths are large, the size and shape of the vessel used may be an important factor in determining the size of the crop when the cultures are undisturbed during the growth period. We use consistently the same size (50 ml.) pyrex Erlenmeyer flasks, and in quantitative tests never rely on heavy crops or long growth periods, with the result that our ability to duplicate results using our thermocouple equipment exceeds in exactness any biological test with which we are acquainted. The use of flasks which are undisturbed during the growth period has a great practical advantage over that of rocker-tubes in a thermostat, as used in the Toronto laboratories, as we are enabled to grow 60–80 cultures simultaneously in one incubator. Handling such numbers in rocker-tubes would, we fear, entail serious expense. It may be emphasised that if the seedings and growths are much larger than those used by us, the relatively small effects produced by inositol alone, for example, may be modified owing to its becoming a limiting factor for growth. Other variations in results are to be expected on the same basis.

The actual data obtained in the tests are given in Table I and the effects of the different nutrilites are summarised in Tables II, III and IV. In addition

to the results indicated in the tables we have experimented sufficiently to convince ourselves that yeast # 578 (2331) of the American type culture collection behaves substantially in the same way as "old process" yeast in that when an inositol-containing medium is used, vitamin B_1 alone has a very definite effect, "pantothenic acid" alone has a smaller, but definite effect, while the effect of the two together is very striking.

Table I. *Yeast crops**.

Addition (to 12 ml. cultures)	Wildiers' yeast		Old process yeast		W. L. Miller's yeast		Narayanan's yeast # 2190		Gebrüder Mayer	Cake Fleischmann
0	0·08	0·09	0·01	0·02	0·05	0·10	0·02	0·03	0·02	0·07
0	0·07	0·09	0·01	0·02	0·05	0·10	0·02	0·03	0·02	0·07
0·1γ I	0·07	0·09	0·02	0·02	0·05	0·11	0·03	0·02	0·02	0·07
1·0γ I	0·07	0·09	0·03	0·03	0·06	0·12	0·03	0·03	0·02	0·07
10γ I	0·09	0·09	0·03	0·03	0·06	0·13	0·03	0·03	0·02	0·07
100γ I	0·09	0·08	0·02	0·04	0·06	0·16	0·03	0·03	0·02	0·07
0·1γ B	0·09	0·07	0·08	0·07	0·05	0·10	0·03	0·03	0·02	0·06
1·0γ B	0·07	0·07	0·10	0·07	0·05	0·10	0·03	0·02	0·02	0·05
10γ B	0·07	0·07	0·11	0·08	0·05	0·10	0·03	0·02	0·02	0·05
0·1γ P	0·08	0·14	0·04	0·03	0·13	0·29	0·03	0·02	0·06	0·14
1·0γ P	0·31	0·44	0·05	0·03	0·61	0·72	0·09	0·03	0·22	0·44
10γ P	0·11	0·33	0·06	0·04	0·71	1·04	0·17	0·09	0·24	0·78
50γ I + 0·1γ B	0·06	0·06	0·06	0·09	0·08	0·19	0·03	0·02	0·02	0·04
50γ I + 1·0γ B	0·05	0·06	0·09	0·09	0·08	0·15	0·04	0·03	0·02	0·05
50γ I + 10γ B	0·05	0·06	0·11	0·11	0·09	0·16	0·04	0·03	0·03	0·04
50γ I + 0·1γ P	0·11	0·17	0·04	0·04	0·17	0·32	0·04	0·04	0·06	0·06
50γ I + 1·0γ P	0·37	0·60	0·05	0·04	0·93	0·96	0·27	0·18	0·64	0·37
50γ I + 10γ P	0·29	0·70	0·14	0·04	1·12	1·44	0·74	0·39	0·77	1·27
1γ P + 0·1γ B	0·28	0·47	0·21	0·32	0·44	0·65	0·12	0·09	0·19	0·44
1γ P + 1·0γ B	0·26	0·46	0·52	0·52	0·38	0·60	0·13	0·10	0·21	0·45
1γ P + 10γ B	0·27	0·41	0·51	0·51	0·39	0·58	0·13	0·09	0·20	0·44
1γ B + 0·1γ P	0·07	0·12	0·21	0·20	0·13	0·21	0·03	0·03	0·06	0·12
1γ B + 10γ P	0·25	0·44	0·62	0·44	0·55	0·81	0·13	0·10	0·24	0·61
50γ I + 0·1γ B + 1γ P	0·38	0·65	0·82	1·17	0·89	0·85	0·25	0·18	0·65	0·54
50γ I + 1·0γ B + 1γ P	0·36	0·58	1·38	1·20	0·88	1·02	0·23	0·22	0·62	0·58
50γ I + 0·1γ B + 0·1γ P	0·06	0·11	0·14	0·16	0·17	0·24	0·04	0·04	0·10	0·05
50γ I + 1γ B + 0·1γ P	0·06	0·09	0·22	0·18	0·20	0·22	0·04	0·04	0·10	0·05

* In the table yeast crops are expressed as mg. of moist yeast per ml. I, B and P stand respectively for inositol, vitamin B_1 and "pantothenic acid" concentrate.

Table II. *The effect of inositol.*

"Strain" used	Added to blank medium alone	Supplement to vitamin B_1	Supplement to "pantothenic acid"	Supplement to vitamin B_1 *plus* "pantothenic acid"
Wildiers'	No effect	No effect	Definite though small increase	Same as without B
Old process	Slight increase	Very slight increase	Very slight increase	Striking increase
W. L. Miller's	Slight increase	About same as without B	Definite increase	Same as without B
Narayanan's # 2190	Very slight increase	Same as without B	Striking increase	Same as without B
Gebrüder Mayer	Not detectable here	No effect	Marked increase in larger growths	Same as without B
Fleischmann Cake	No effect	No effect	Marked increase in larger growths	About same a without B

Table III. *The effect of vitamin B_1.*

"Strain" used	Added to blank medium alone	Supplement to inositol	Supplement to "pantothenic acid"	Supplement to inositol *plus* "pantothenic acid"
Wildiers'	No effect	Slightly inhibitory	Variable, slight	Slightly inhibitory
Old process	Definite increase	Definite increase	Most striking increase	Most striking increase
W. L. Miller's	No effect	Slight increase	Slightly inhibitory	No effect
Narayanan's #2190	No effect	Increase questionable	Slight increase	Practically no effect
Gebrüder Mayer	No effect	No effect	Inhibitory questionable	Inhibitory questionable
Fleischmann Cake	Slightly inhibitory	Slightly inhibitory	No effect	No effect

Table IV. *The effect of "pantothenic acid."*

"Strain" used	Added to blank medium alone	Supplement to inositol	Supplement to vitamin B_1	Supplement to inositol *plus* vitamin B_1
Wildiers'	Striking increase	Striking increase	Same as without B	Same as without B
Old process	Definite slight increase	Slight increase	Striking	Most striking
W. L. Miller's	Striking increase	Striking increase	Good growth, less than without B	Same as without B
Narayanan's #2190	Definite increase	Striking increase	Definite increase	Practically same as without B
Gebrüder Mayer	Striking increase	Striking increase	Same as without B	Same as without B
Fleischmann Cake	Striking increase	Striking increase	About same as without B	About same as without B

It is to be noted that in the summaries (Tables II, III and IV) the results as tabulated were, in the case of the first four yeasts, obtained twice in independent experiments each involving two to three cultures. Since there is no disagreement between the different experiments (except possibly in the case of a single culture), we feel that the differences are significant, and that the five pure cultures listed represent different species of organisms. As they have been grown on the same medium for years, the differences can hardly be accounted for on any other basis. The individual behaviour of "old process" (or #578) yeast is very striking and cannot be questioned. The difference in behaviour between the yeast of Miller and that of Wildiers, particularly with respect to the response toward inositol and crystalline vitamin B_1 together, is, in our opinion, significant. Wildiers' yeast has also throughout a period of years been peculiarly susceptible to the effect of toxic substances which appear in extracts. This was particularly apparent in the experiments of Williams, R. J. and Truesdail [1931]. The fact that these two yeasts react similarly towards the crude preparations of Miller *et al.* [1933] does not constitute proof of the identity of the active ingredients of the extracts, though from our experiments there remains no doubt that both "pantothenic acid" and inositol are common factors for the two yeasts.

There is no doubt in our minds that there is an unknown basic factor effective for Wildiers' yeast which is not vitamin B_1. We have repeated some of the

fractional electrolysis experiments of Williams and Truesdail on rice bran extracts and confirm their findings regarding the supplementary action of acidic and basic components of the extract. We find also that the basic component and our concentrated "pantothenic acid" supplement each other. However, there is a significant difference in our results in that the acid factor alone (containing "pantothenic acid") now causes very distinct stimulation, whereas in the earlier experiments, it did not. We attribute the difference to the probability that in the earlier experiments toxic material (which was constantly encountered in the more acid cells) was not removed completely, and hence masked what stimulating effect the acidic component ("pantothenic acid") would have had.

A careful analysis of the results given in Table I reveals the fact that sometimes the nutrilites which, under one set of conditions promote increased growth, under a different set of conditions may inhibit growth. We believe there is some evidence that to promote most rapid growth some yeasts require a "balance" between the different stimulating factors. This matter requires further study.

That inositol is a factor in the growth stimulation of all the yeasts studied is a striking fact, and Miss Eastcott working in Dr Miller's laboratory deserves full credit for the discovery. That several other workers have not noted a positive effect of inositol, has been due in part to the reasons outlined by Miller *et al.* [1933], and also to the lack of proper supplements. In our laboratory an important factor has been the fact that we have consistently dealt with small growths and under such conditions the effect of inositol alone or in combination is very small, except in the case of the yeast from the Toronto laboratories which we found to be affected [Williams, R. J. and Bradway, 1931]. To be effective inositol evidently must be present in much larger amounts than the other nutrilites. We are basing this statement partly on unpublished experiments on "Gebrüder Mayer" yeast and partly on the results obtained in the Toronto laboratories.

The striking effect of crystalline vitamin B_1 as a supplement to "pantothenic acid" (also alone) in stimulating "old process" yeast shows that the senior author's original suggestion as to the probable identity of "bios" (for "old process" yeast) and the water-soluble B vitamin [Williams, R. J., 1919] was not wholly without substantial basis. There are, however, other complications which have previously been noted [Williams, R. J. and Roehm, 1930].

The results with "pantothenic acid" on the different yeasts are much more striking than we anticipated, especially the marked effects which it has when used without any supplement. It is apparent to us that this material stands alone in so far as its ability in minute doses to stimulate different strains of yeast is concerned.

Since our results and claims in this respect are striking and must be confirmed by other workers, our laboratory will be glad to furnish interested investigators in this field with samples of our concentrates in order that they may carry out whatever confirmatory tests on yeast they may desire to make.

Summary.

1. The different strains of so-called *Saccharomyces cerevisiae* used in the studies of Wildiers, R. J. Williams, W. L. Miller, Eddy and Narayanan have been studied.

2. Inositol is found to be at least a detectable factor in the growth-stimulation of all the yeasts investigated. Alone, it in general has very little effect, but in conjunction with the other substances mentioned, it may cause a striking

increase. The amounts necessary to produce effects are relatively large (1γ to 10γ per ml. of medium).

3. Crystalline vitamin B_1 has very little effect on any of the yeasts except "old process" yeast on which the original work of the senior author was done and on yeast 578, also investigated in his laboratory. As little as $0{\cdot}008\gamma$ per ml. has a distinct effect alone on these yeasts, and in conjunction with pantothenic acid, the effect of doses of this size and larger is very striking.

4. A highly concentrated "pantothenic acid" preparation was found to be effective alone in doses of $0{\cdot}008\gamma$ per ml. upwards, on all the yeasts investigated. This effect was in general enhanced more or less either by inositol or vitamin B_1 or both. In some cases both the individual and supplementary effects are most striking. The different yeasts, in most cases, show characteristic responses to the different materials.

5. The results are so conclusive and striking that we feel that the "bios question" is to a considerable extent answered. No single (unknown) substance is wholly responsible for yeast growth stimulation. The three substances studied play important rôles, of which that of "pantothenic acid" is outstanding.

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CCXLVII. THE MECHANISM OF BACTERIAL ANAEROBIOSIS.

III. THE OXIDATION-REDUCTION POTENTIAL OF THE BUTYRIC BACILLUS IN PRESENCE OF SULPHYDRYL COMPOUNDS.

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It has been shown in a previous publication [1933] that anaerobic bacteria can be cultivated under aerobic conditions in symbiosis with aerobes or in presence of certain compounds which act as hydrogen acceptors. Among them sulphydryl compounds may be mentioned which were found to be active not only in the oxidised but also in the reduced form. This is no doubt due to the fact that during growth of the bacteria one of the forms is partially converted into the other one no matter which of them was added to the culture-medium. According to Hopkins [1931], some dynamic equilibrium seems to exist in the tissues between the oxidised and reduced forms of glutathione, and this may be regarded as a support to the behaviour of the cysteine-cystine system.

We are still quite ignorant with regard to the mechanism of action of sulphydryl compounds on the growth of anaerobic bacteria though several attempts have been made to throw some light upon the problem. Hosoya and Kishino [1925], and independently of them Quastel and Stephenson [1926] have shown that aerobic growth of strict anaerobes can proceed in the presence of sulphydryl compounds. According to these authors, the action of sulphydryl compounds consists not only in removal of free oxygen through oxidation of these compounds but also in the establishment of a certain level of the reduction potential which is indispensable for the growth of the anaerobic bacteria. This point of view is supported by Aubel *et al.* [1929] who have shown colorimetrically that growth of strict anaerobes can proceed only in a medium the redox potential of which varies from $r_H 0$ to $r_H 12$. Clark [1924] has also advanced the idea that a certain reducing potential of the medium is indispensable for the growth of anaerobic microbes, and Fildes [1929] and Knight and Fildes [1930] have shown colorimetrically that germination of the spores of *Bacillus tetani* becomes possible only at a certain level of the redox potential of the medium which should not exceed the value of $E_H = +0{\cdot}01$ volt.

Plotz and Geloso [1930] maintained the reduction potential of the medium at a definite level by introducing into it certain oxidants or reductants. Cultivation of various anaerobic bacteria in the above media has shown that growth of the culture can proceed only within the range of r_H lying between 0 and 14. The potential of the medium whatever be its initial value is reduced by growth of the bacteria to r_H 5·5 irrespectively of the species used.

Establishment of a definite level of the reduction potential has been sometimes regarded, however, not as a factor which is necessary for the growth of

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for $E_H = +0{\cdot}01$ volt *read* $E_H = +0{\cdot}11$ volt

anaerobic microbes but as a result of this growth. In this connection a paper by Pasteur [see Plotz and Geloso, 1930] should be mentioned in which the reducing capacity of the butyric bacillus was shown by decoloration of indigo-blue. Among more recent publications are those of Tarozzi [1905] and Potter [1911] on *Bacillus coli*, of Gillespie [1920] on soil bacteria, and finally that of Lepper and Martin [1930]. Some authors however do not regard establishment of a certain negative oxidation-reduction potential as a necessary condition of bacterial growth. Coulter and Isaaks [1929] by passing a finely divided current of oxygen into the culture-medium raised the reduction potential of the medium to a positive level which did not effect any inhibition of growth. Hewitt [1930] regards the bacteria themselves as responsible for the negative potential of the medium as the result of metabolic processes on the cell-surface.

In a new publication by Quastel and Wooldridge [1929] Quastel gives up his former concept of the negative potential as an indispensable factor of anaerobic growth. According to the new concept of Quastel, much more weight should be attached to the chemical composition of the medium and to the nature of catalysts operating on the surface of the bacterial cell, while the potential itself is regarded as a factor of secondary importance.

It will be seen from the above that the problem of bacterial anaerobiosis is yet an open one, though its theoretical as well as practical importance can hardly be doubted. Solution of this problem promises to give an insight into bacterial metabolism in general and into the mechanism of formation of bacterial products such as toxins and enzymes, or spore formation *etc.* It would also render it possible to change the bacterial metabolism at will and to study the possibility of cultivation of anaerobic microbes on synthetic media.

In the present publication an attempt has been made to find out the correlation between the part played by sulphydryl compounds in the aerobic growth of anaerobic microbes and the oxidation-reduction potential of the system. The latter was determined electrometrically by means of the usual technique.

Methods.

The butyric bacillus (a pure culture of *B. saccharobutylicus*, anaerobic strain) was cultivated in full test-tubes of 200 ml. capacity containing 100 ml. of culture-medium. The rubber-stopper had four holes through two of which the platinum electrode and KCl-agar bridge were introduced while the other two served for passing the stream of purified nitrogen. As culture medium a 2 % sugar bouillon was used, or a bouillon fermented by yeasts in which the content of peptone amounted to 2 % instead of the 1 % usually employed, or a 0·25 % sugar bouillon with the same content of peptone. Reduced glutathione, cysteine or cystine was added to the medium so that the final concentration of the substance amounted to 0·02 %. The above concentrations of glucose and of sulphydryl compounds were low enough not to influence the redox potential of the medium. The p_H of the medium was kept at 7·0–7·1, and the temperature of the thermostat at 37°. Experiments were carried out mostly under anaerobic conditions while occasional aerobic experiments served to follow up the reduction potential in the presence of air. Anaerobiosis was insured by passing through the test-tube a stream of nitrogen freed from traces of oxygen by passing it through a heated quartz tube filled with metallic copper. The potential was followed up electrometrically every $\frac{1}{2}$–1 hour in the course of 6–7 days, and occasionally up to 3 weeks.

Results.

The results of the experiments are illustrated by the curves of Fig. 1.

It will be seen from the data obtained that both the pure medium and the medium containing the bacterial culture are represented by the same type of curve either under anaerobic or aerobic conditions. It will be noted also that the anaerobic potential is more negative than the aerobic, whereas in the presence of

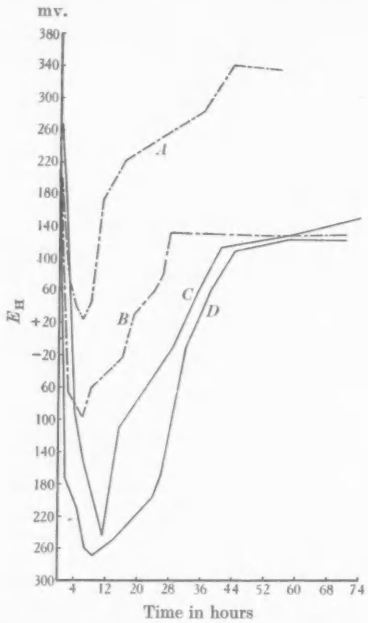

Fig. 1. Variation of E_H with time. *A*, 2 % sugar medium; aerobic. *B*, 2 % sugar medium; anaerobic. *C*, 2 % sugar medium + bacteria; aerobic. *D*, 2 % sugar medium + bacteria; anaerobic.

bacteria it shifts still more to the negative side. Growth of bacteria in a sugar-containing medium is initiated within the first hour after inoculation. This is accompanied by an abrupt fall of the potential curve, while on the next day the potential of the pure medium, and two days later that of the culture, becomes more positive reaching the initial value or even exceeding it, though growth of the bacteria still continues. The character of the curves does not render it possible to discriminate between the behaviour of the medium itself and that of the culture, the difference, if any, being a quantitative one.

The 2 % sugar medium was replaced therefore by one containing less sugar, *viz.* one containing 0·25 % sugar in a bouillon which had been previously fermented with yeast and contained 2 % of peptone. The results obtained with this medium are illustrated by the curves of Fig. 2.

It will be noted that there is no drift to negative potential in the bouillon itself either containing sulphydryl compounds or not. The aerobic potential

however is more positive than the anaerobic. If however a culture of bacteria is introduced into the medium (and also in symbiosis with sarcina) the potential shifts to the negative in the course of bacterial growth, attaining a value which lies close to $E_H = -280$ mv. This level of potential remains constant for a considerable period of time as well as the p_H of the medium.

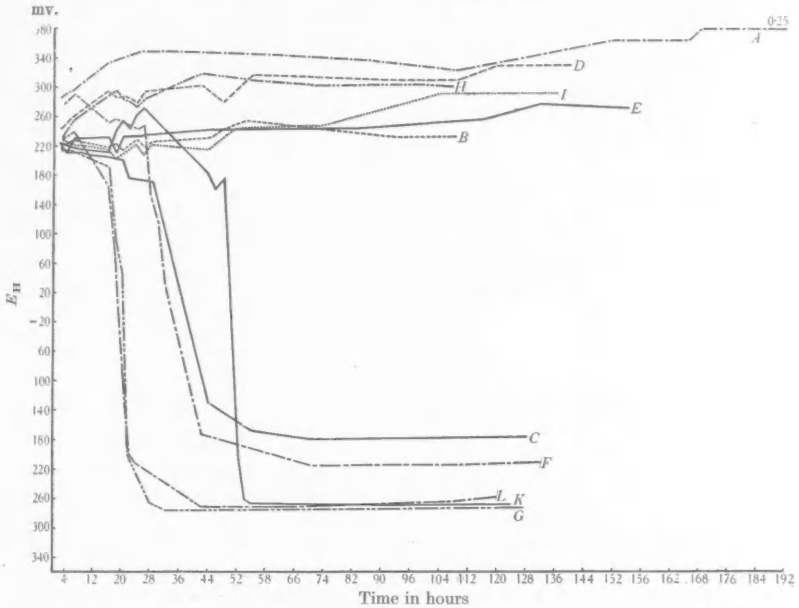

Fig. 2. Variation of E_H with time. *A*, 0·25 % sugar bouillon; aerobic. *B*, 0·25 % sugar bouillon; anaerobic. *C*, 0·25 % sugar bouillon + bacteria; anaerobic. *D*, 0·25 % sugar bouillon + cysteine; aerobic. *E*, 0·25 % sugar bouillon + cysteine; anaerobic. *F*, 0·25 % sugar bouillon + cysteine + bacteria; aerobic. *G*, 0·25 % sugar bouillon + cysteine + bacteria; anaerobic. *H*, 0·25 % sugar bouillon + glutathione; aerobic. *I*, 0·25 % sugar bouillon + glutathione; anaerobic. *K*, 0·25 % sugar bouillon + glutathione + bacteria; aerobic. *L*, 0·25 % sugar bouillon + glutathione + bacteria; anaerobic.

No drift of potential has been noted in the pure medium, which is due to the fact that it contains a rather low concentration of glucose and of sulphydryl compounds. The oxidation-reduction curves obtained in bacterial cultures which contain cysteine or glutathione are nearly identical: the drop of the anaerobic cysteine curve precedes that of the glutathione curve, though the latter becomes stabilised at a more negative level than the former. The data of Table I illustrate the rate of fall of potential during bacterial growth.

A concentration of 0·25 % glucose in the medium suffices to induce anaerobic growth of the butyric bacillus, which sometimes may become possible even under aerobiosis when cultivating the organism in a deep layer of liquid. In order to study the part played by sulphydryl compounds it was necessary therefore to cultivate this organism in a medium absolutely free from glucose.

The curves of Fig. 3 refer to the new culture medium (bouillon, the broth of which had been fermented with yeast). In this medium the butyric bacillus

Table I.

	E_H mv.	Time		E_H mv.	Time
Cysteine + bacteria	+189	9 a.m.	Glutathione + bacteria	+164	9 a.m.
	+ 97	11 a.m.		+ 67	11 a.m.
	+ 40	11.30 a.m.		+ 45	11.05 a.m.
	+ 12	12 noon		+ 9	11.25 a.m.
	− 1	12.05 p.m.		− 42	12 noon
	− 56	12.35 p.m.		− 62	12.30 p.m.
	− 96	1 p.m.		−106	1 p.m.
	−162	2 p.m.		−169	2 p.m.
	−199	3 p.m.		−197	3 p.m.

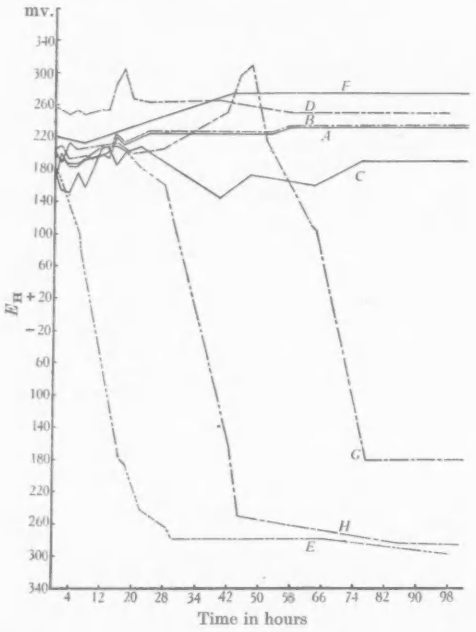

Fig. 3. Variation of E_H with time. *A*, Fermented bouillon; anaerobic. *B*, Fermented bouillon + bacteria; anaerobic. *C*, Fermented bouillon + cysteine; anaerobic. *D*, Fermented bouillon + cystine; anaerobic. *E*, Fermented bouillon + cysteine + bacteria; anaerobic. *F*, Fermented bouillon + glutathione; anaerobic. *G*, Fermented bouillon + glutathione + bacteria; aerobic. *H*, Fermented bouillon + glutathione + bacteria; anaerobic.

does not grow even under anaerobiosis; this is shown by the curve running nearly parallel with that of the culture medium itself. However the addition of 0·02 % of sulphydryl compound to the medium induces growth of bacteria both aerobically and anaerobically; this is shown by the course of the curves. This illustrates the part played by sulphydryl compounds in the aerobic growth of anaerobic microbes. In presence of a negligible amount of a sulphydryl compound the bacteria are enabled to react with the medium by decomposing the nutriment supplied by the medium and oxidising it, whilst the energy liberated by oxidation is used up for synthetic processes going on in the cells. The rôle of the sulphydryl compounds seems therefore to consist in promoting the inter-

action between the bacteria and the medium. Little is known as yet about the nature of this activation but it will be noted that sulphydryl compounds are known to activate various enzymic processes, *e.g.* proteolysis and conversion of methylglyoxal into lactic acid and also to exert a certain influence upon the Pasteur-Meyerhof reaction through which some correlation between respiration and fermentation is maintained. It seems probable that we are dealing here with an activation of some processes which are necessary for establishment of oxidation-reduction equilibria.

Bamberger *et al.* [1933] have shown indeed that the irreversible system of pyruvate-lactate becomes reversible in presence of an enzyme and of a reversible system consisting of methylene blue-leucomethylene blue. Its E_H can be correctly determined, and it proved to fall into the same negative range of potential as that found in the above systems. A tentative suggestion may therefore be made that in the systems reported above a similar redox equilibrium might be established, the more so as pyruvic acid is known to appear in the fermentation processes as an intermediate product.

Cultivation of anaerobic microbes on synthetic media would be an important contribution to the study of the mechanism of anaerobiosis, and application of the redox potential method to the study of general bacterial metabolism seems to be very suggestive.

Summary.

1. Sulphydryl compounds promote the growth of anaerobic bacteria either under aerobiosis or anaerobiosis.

2. Growth of the bacteria is accompanied by a drop of the redox potential, which acquires a definite value within two or three days in a medium containing cysteine or glutathione. The anaerobic potential corresponds to $E_H = -280$ mv. while the aerobic potential is more variable. In presence of glutathione it varies from -280 to -180 mv.; in presence of cysteine it amounts to -220 mv.

3. The drop of the anaerobic potential in presence of cysteine either precedes that of the glutathione medium or coincides with it, while under aerobiosis the former occurs first.

The courses of both the curves were found to be identical and this suggests that the mechanism of action is similar in both cases. In absence of bacteria and in a medium containing microbes which do not grow no drop of potential has been found.

4. Growth of bacteria is probably not due to establishment of a negative reduction potential. It is initiated prior to this when the potential is still positive and in a sugar-containing medium continues even at a positive potential level.

5. Growth of bacteria in the presence of sulphydryl compounds and of sugars should not be attributed to the reduction potential of the medium, since the negative potential does not become established in a medium containing small amounts of these substances in absence of bacteria. The drop of potential is due to some interaction which takes place between the bacteria and the medium in the course of metabolic processes going on in the bacterial cell.

6. The composition of the medium (sugar or sulphydryl compounds) influences the course of single phases of the bacterial metabolism, this being shown by the shape of the corresponding curves (see Figs. 1 and 2).

7. Sulphydryl compounds seem to exert some peculiar activating effect upon the interaction between the microbes and the medium as a result of which certain coupled reversible systems of the type lactate-pyruvate might appear.

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CCXLVIII. ON THE MECHANISM OF THE FORMATION OF CHOLEIC ACIDS.

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It is well known since the work of Wieland and Sorge [1916] that desoxycholic acid may combine with a large number of organic substances acting as centres of co-ordination, so as to form molecular compounds, the so-called choleic acids. This phenomenon probably plays an important part in the metabolism of water-insoluble substances. Rheinboldt and co-workers [1927; 1929] and Sobotka and Goldberg [1932] have established a number of principles as to the arrangement of desoxycholic acid molecules round aliphatic mono- and di-carboxylic acids. In proportion to the length of the fatty acid chain the co-ordination numbers increase by certain intervals. The co-ordination number of acetic acid is 1, of propionic acid 3, of butyric to octanoic acid 4, of nonanoic to tetradecanoic acid 6 and of pentadecanoic and higher acids 8.

We have studied the influence exercised by changes in the chain structure of fatty acids upon their ability to act as co-ordination centres of choleic acids. A few similar experiments with lower fatty acids have been described by Sobotka and Goldberg [1932].

A systematic study of a series of isomeric fatty acids with branched chains would lead to a better understanding of the mechanism of the formation of choleic acids, a reaction which is rather unusual in organic chemistry. It also would be of importance in regard to the question of the absorption of fatty acids with branched carbon chains.

Since one of us (Abel) will not be able to participate in the further investigation of these problems, we are here describing the results obtained up to the present time. Further material will be communicated in a later paper.

Two ways exist of studying the composition of choleic acids. (1) The choleic acid is prepared by direct synthesis. The molecular composition of the compound is either calculated from the acid equivalent determined by titration or after removal of the desoxycholic acid in the form of its insoluble xylene compound the fatty acid component is estimated by titration [Wieland and Sorge, 1916]. (2) Determinations are made of the melting- and thawing-points of mixtures of the two components in different proportions. From the melting-thawing diagram thus obtained conclusions can be drawn as to the existence of a molecular compound in the particular case and as to its composition [Rheinboldt, 1925; Sobotka and Goldberg, 1932].

In the case of the fatty acids with branched chains examined by us we have almost always observed that the choleic acids formed were extremely labile, much more so than the choleic acids of the corresponding normal fatty acids, for which reason we have often had to use the melting-thawing diagram in order to establish the existence and molecular composition of co-ordination

compounds. Characteristic differences could be noted in the stability of choleic acids with α- and β-substituted fatty acids, the latter being more stable than the former. The fact that branched chain fatty acids as compared with normal fatty acids are less apt to form choleic acids and therefore are probably less easily absorbed perhaps explains the absence of such acids in nature.

The fatty acids that have hitherto been examined by us are summarised in Table I. A study of this table shows that in the case of the acids which we

Table I. *Formation of choleic acids with fatty acids.*

No. of exp.	Substance	Co-ordination number established by: Crystallised choleic acid	Co-ordination number established by: Diagram	Co-ordination number of corresponding normal fatty acid
1	α-Butylhexanoic acid	4	4	6
2	α-Ethyloctanoic acid	0	2	6
3	β-Methylnonanoic acid	6	–	6
4	α-Ethyldecanoic acid	4	–	6
5	α-Methylundecanoic acid	4	–	6
6	α-Ethylhexadecanoic acid	8	–	8
7	α-Dodecyltetradecanoic acid	0	4 and 8	8
8	Trichloroacetic acid	0	–	1
9	β-Iodopropionic acid	0	0	3
10	α-Bromopentanoic acid	0	0	4
11	α-Bromo*iso*pentanoic acid	0	0	4

0 = no compound obtained. – = not examined.

examined not only were the choleic acids formed relatively unstable but the co-ordination numbers were often lower than those of the straight chain isomerides. From this one may infer that the grouping within the carbon chain that is responsible for co-ordination has been changed. The methylene group in the α-position seems to be particularly important, as is shown by Exp. 3, when compared, for instance, with Exp. 5: while β-methylnonanoic acid has the normal co-ordination number 6, the co-ordination number of α-methylundecanoic acid containing two more methylene groups is only 4. On the other hand, the relation between the length of the main chain and that of the side-chain also seems to exert an important influence. The longer the main chain, as compared with the side-chain, the more similar is the behaviour of the fatty acid to that of the corresponding normal compound. On computing the relation between the two chains attached to the same carbon atom by dividing the molecular weight of the longer by that of the shorter chain one finds as quotients for Exps. 1–7 (see Table I): 1, 2·9, 6·3, 3·9, 8·5, 6·4, 1. With the sole exception of Exp. 5 all acids, the quotients of which exceed 6·3—that is where one chain preponderates—have a normal co-ordination number. The acids having smaller quotients show a diminution of their co-ordinative power.

With the halogenated fatty acids which we examined we did not observe any formation of choleic acids at all. This may be due to the introduction of a relatively very heavy constituent into the chain. In halogenated fatty acids however where the halogen does not outweigh the carbon chain normal co-ordination numbers are observed. Dibromobehenic acid $C_{22}H_{42}O_2Br_2$ was found by Rheinboldt *et al.* [1927] to have the co-ordination number 8.

The fact that relatively small changes in the molecule of a chemical compound may have a far-reaching influence upon its co-ordinative power is reminiscent of certain immuno-chemical phenomena [*cf.* Landsteiner, 1933].

Experimental.

A. *Material.*

The halogenated acids which we examined were purified preparations of Schering-Kahlbaum. The fatty acids were all prepared by synthesis.

(1) *α-Butyl-n-hexanoic acid* [Levene and Cretcher, 1918], B.P. 108–110°/2 mm. was prepared from ethyl dibutylmalonate, B.P. 111–114°/3 mm., which was converted into dibutylmalonic acid, M.P. 164–165° (corr.).

(2) *α-Ethyl-n-octanoic acid* [Dox, 1925], B.P. 135–137°/3 mm. was prepared from ethyl ethylhexylmalonate, B.P. 113–115°/3 mm. [Dox, 1924].

(3) *β-Methyl-n-nonanoic acid* [Levene and Taylor, 1922], B.P. 115–120°/3 mm. was prepared from ethyl 1-methylheptylmalonate, B.P. 131–133°/3 mm.

(4) *α-Ethyl-n-decanoic acid* [von Braun and Teuffert, 1929], B.P. 151–152°/3 mm. was prepared from ethyl ethyloctylmalonate, B.P. 142–143°/4 mm.

(5) *α-Methyl-n-undecanoic acid.* To a solution of 0·66 g. Na in 25 ml. of absolute alcohol 6·0 g. of ethyl methylmalonate and 7·3 g. of freshly purified nonyl iodide (B.P. 75°/4 mm.) were added. After boiling the mixture for 8 hours the alcohol was partly removed by distillation and the residue diluted with acidified water and extracted with ether. The ethereal solution was washed with dilute thiosulphate solution and water, dried and concentrated. The remaining oil was distilled *in vacuo*. 5·6 g. of ethyl methylnonylmalonate (64 % of the theoretical yield) were obtained as a colourless oil, B.P. 147–148°/5 mm. (Found: C, 67·75; H, 10·45 %. $C_{17}H_{32}O_4$ requires C, 67·94; H, 10·74 %.) The ester was saponified according to the method of Adams *et al.* [1928]. Methylnonylmalonic acid was obtained by crystallisation from benzene in the form of small plates (3·2 g.), M.P. 94–95°. (Found: C, 63·62; H, 9·54 %. $C_{13}H_{24}O_4$ requires C, 63·87; H, 9·90 %.) By heating *in vacuo* CO_2 was eliminated and α-methylundecanoic acid was obtained as an oil which solidified on cooling. The acid had B.P. 125–127°/6 mm. (Found: C, 72·07; H, 11·89 %. Mol. wt. by titration 198·5. $C_{12}H_{24}O_2$ requires C, 71·94; H, 12·09 %. Mol. wt. 200·2.)

(6) The syntheses of *α-ethyl-n-hexadecanoic acid* and α-n-*dodecyl*-n-*tetradecanoic acid* have already been described [Chargaff, 1932].

B. *Choleic acids.*

In order to prepare the choleic acids the two components were dissolved in a small quantity of hot alcohol. In all cases where stable choleic acids were obtainable, crystals separated on cooling and were then recrystallised from 2 to 4 times their weight of absolute alcohol. The desoxycholic acid used was a pure preparation of Riedel-de Haën which melted at 174°.

The ratio fatty acid : desoxycholic acid was either determined by the xylene or xylene-calcium method [Wieland and Sorge, 1916; Rheinboldt *et al.*, 1927] or by titration of the acid equivalents of the choleic acids with alcoholic KOH and α-naphtholphthalein as indicator.

The thawing-melting diagrams were made according to the indications of Rheinboldt [1925] and Sobotka and Goldberg [1932]. In the case of liquid substances the mixtures were prepared by vigorous shaking of the melted components contained in closed thin-walled tubes in an oil-bath at 195°. After cooling the contents were thoroughly ground.

(1) *α-Butyl-n-hexanoic acid–tetracholeic acid.* 0·29 g. of α-butyl-*n*-hexanoic acid and 4 g. of desoxycholic acid, dissolved in 6 ml. of hot absolute alcohol, gave, after four recrystallisations from a little alcohol, 1·5 g. of fine plates which melted at 147–148°. (Found: acid equivalent 350·4, 348·2. $[C_{24}H_{40}O_4]_4.[C_{10}H_{20}O_2]$ requires acid equivalent 348·3.)

The thawing-melting diagram of mixtures of desoxycholic and butylhexanoic acids is reproduced in Fig. 1. At 9·7 % of butylhexanoic acid both curves show a maximum

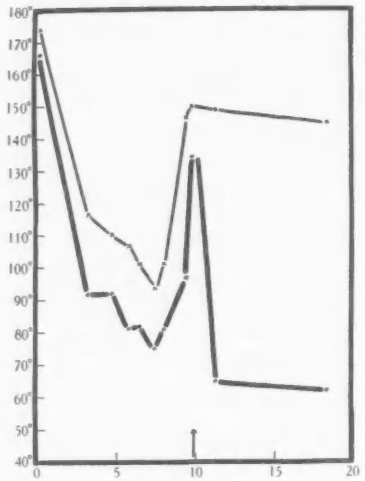

Fig. 1.

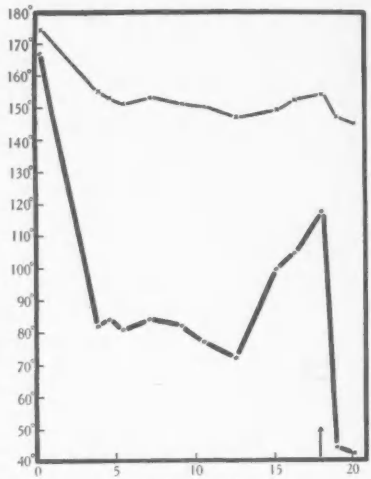

Fig. 2.

Fig. 1. System α-butyl-*n*-hexanoic acid + desoxycholic acid.

Fig. 2. System α-ethyl-*n*-octanoic acid + desoxycholic acid.

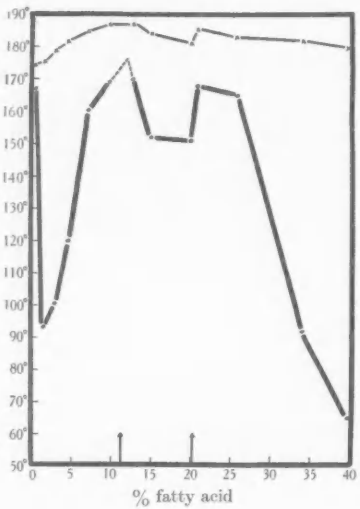

Fig. 3.

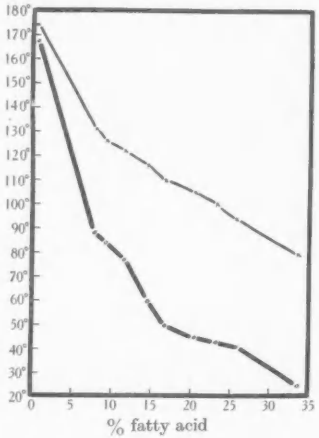

Fig. 4.

Fig. 3. System α-*n*-dodecyl-*n*-tetradecanoic acid + desoxycholic acid.

Fig. 4. System β-iodopropionic acid + desoxycholic acid.

corresponding to a compound of the co-ordination number 4 (required: 9·8 % $C_{10}H_{20}O_2$) and M.P. 150°.

(2) *α-Ethyl-n-octanoic acid–dicholeic acid.* A crystalline choleic acid could not be obtained. As Fig. 2 shows, there exists a molecular compound $[C_{24}H_{40}O_4]_2.[C_{10}H_{20}O_2]$ of M.P. 154° at 18·0 % ethyloctanoic acid (required: 17·9 %).

(3) *β-Methyl-n-nonanoic acid–hexacholeic acid.* 0·29 g. fatty acid and 4 g. desoxycholic acid in 10 ml. absolute alcohol gave 2·5 g. of a choleic acid which on recrystallisation from alcohol was obtained in beautiful platelets of M.P. 170·5°. For this compound, which was much more stable than most of the choleic acids examined, a molecular ratio of 1 : 6 was found. (Found: acid equivalent 358·3, 358·4, fatty acid (xylene-calcium method) 6·96, 7·03 %. $[C_{24}H_{40}O_4]_6.[C_{10}H_{20}O_2]$ requires acid equivalent 360·8, fatty acid 6·79 %.)

(4) *α-Ethyl-n-decanoic acid–tetracholeic acid.* From 0·4 g. fatty acid and 2·8 g. desoxycholic acid in 12 ml. absolute alcohol a crystalline choleic acid was obtained which after two recrystallisations had M.P. 176°. The co-ordination number was 4. (Found: acid equivalent 355·0, 355·4. Required by $[C_{24}H_{40}O_4]_4[C_{12}H_{24}O_2]$ acid equivalent 353·9.)

(5) *α-Methyl-n-undecanoic acid–tetracholeic acid.* From 0·2 g. fatty acid and 3·1 g. desoxycholic acid in 10 ml. absolute alcohol crystals were obtained which had M.P. 179° and the molecular ratio 1 : 4. (Found: acid equivalent 354·3, 353·2. $[C_{24}H_{40}O_4]_4.[C_{12}H_{24}O_2]$ requires acid equivalent 353·9.)

(6) *α-Ethyl-n-hexadecanoic acid–octacholeic acid.* From 0·5 g. fatty acid and 5 g. desoxycholic acid in 20 ml. absolute alcohol after three recrystallisations 2 g. of small plates were obtained which had M.P. 178–179°. The choleic acid showed the normal ratio 1 : 8. (Found: fatty acid (xylene-calcium method) 8·31, 8·18 %. Required by $[C_{24}H_{40}O_4]_8.[C_{18}H_{36}O_2]$ fatty acid 8·31 %.)

(7) The synthesis of a series of hexacosanoic acids with branched carbon chains has been described by one of us [Chargaff, 1932]. The experiments undertaken with a view to prepare the choleic acids of this series led to a number of high-melting optically active crystalline products to which however no rational co-ordination numbers could be assigned in most of the cases. This anomaly may have the following causes: (i) The fatty acid component being only sparingly soluble in alcohol, recrystallisations from alcohol bring about the total decomposition of the molecular compound and the pure fatty acid crystallises. (ii) The high-molecular fatty acids with branched chains apparently form choleic acids in multiple proportions. The thawing-melting diagram of α-dodecyltetradecanoic and desoxycholic acids (Fig. 3), and particularly the thawing-point curve, demonstrates the existence of two choleic acids with the molecular ratios 1 : 4 and 1 : 8. The formation of two different choleic acids by the same substance has already been described for *d*-camphor [Rheinboldt, König and Flume, 1929], for *n*-butyric acid and for ethyl butyrate [Sobotka and Goldberg, 1932].

(8) With *halogenated lower fatty acids* we could not obtain any choleic acids at all, nor did the thawing-melting diagrams reveal the existence of co-ordination compounds. As an example the diagram obtained with *β-iodopropionic acid* is shown in Fig. 4.

Summary.

A number of fatty acids with branched carbon chains and of halogenated fatty acids were studied in regard to their ability to form choleic acids. In comparison with the choleic acids of normal fatty acids essentially different results were obtained, since the co-ordinative power of fatty acids is considerably changed by the introduction of substituent groups. These phenomena are discussed with regard to the choleic acid problem.

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CCXLIX. CATHEPSIN IN RATS WITH TRANSPLANTABLE CANCER.

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In a recent paper Waldschmidt-Leitz, McDonald and co-workers [1933] reported on the general distribution and state of activity of cathepsin in transplantable rat cancers of various stages of development. It was found that with the ageing of the tumour (increase of necrosis) the cathepsin content decreases, indicating that the cathepsin occurs chiefly in the parenchymal tissue rather than in the necrotic portions of the tumour. At the same time a number of cases were met in which the cathepsin contents of livers of cancerous animals were considerably altered from that of normal rat liver.

These findings suggested further studies of the general distribution of cathepsin in the organs of healthy, cancerous and cancer-resistant rats. Such studies disclose that in normal rats the cathepsin content of the liver (measured as full activity) is almost constant from animal to animal, while the muscular system of the same rats is practically free from cathepsin (Table II). In rats with transplantable cancer, however, there is a significant increase of the cathepsin concentration (as judged by the activity) in the liver, while coincidentally a considerable amount of this enzyme appears in the muscle tissue. Edlbacher and Merz [1927] have found that arginase shows a similar behaviour, occurring in large amounts in the muscle of animals with transplantable cancer but not in normal muscle. In this increased arginase activity these authors see a manifestation of a specific growth factor which, like the disturbed glycolysis (known through the investigations of Warburg [1926]), is believed to be characteristic of malignancy. In view of our experimental findings, the increased catheptic activity of liver and muscle must likewise be considered as a manifestation of the growth factor.

In this connection, it was of interest to study the cathepsin distribution in the organs of cancer-resistant rats as well (rats in which transplants failed to grow). It was found that these animals have a particularly high cathepsin content in liver and muscle, as compared with the normals (usual laboratory albino rats). Koehler [1934] has likewise reported that the kidney-phosphatase of cancer-resistant rats is considerably higher than that of the normal kidney. It seems to be characteristic of cancer-resistant organisms that they have a high and uniform enzymic metabolism.

A number of experiments were carried out to test further the original conclusion of Waldschmidt-Leitz, McDonald and co-workers [1933] that tumour-cathepsin occurs chiefly in the rapidly growing parenchymal tissue. As shown in Table III, analysis of histologically homogeneous portions of tumour material confirmed the previous findings that cathepsin occurs chiefly in the rapidly growing parenchymal tissue, to a less extent in the fibrous tissue and is practically

absent from the necrotic portions of the tumour. To a certain extent, arginase shows the opposite picture.

The physiological interpretation of these findings is opposed to the hypothesis set up several years ago by Waldschmidt-Leitz and Schaeffner [1930] on the activity of proteolysis in tumours. According to this hypothesis, the increased proteolytic activity of the tumour cell (which Waldschmidt-Leitz considered as proved) is due to an activation of the intracellular proteinase (cathepsin) by increasing amounts of reduced glutathione. The increased formation of reduced glutathione is ascribed to a preponderance of reduction processes in the tumour, due to a fall in oxidation-reduction potential and decrease of respiration. This relationship between proteolysis and respiration in the cell he believed to be responsible for the necrosis occurring in the tumour tissue.

The hypothesis of Waldschmidt-Leitz is certainly not correct in its present form; in fact, it has been questioned by other investigators [Krebs, 1931; Kleinmann and Werr, 1931] and recently also by Waldschmidt-Leitz, McDonald and co-workers [1933]. The results reported in the present paper not only fail to support this hypothesis, but also indicate that the action of cathepsin during the growth of the tumour may become exerted in the direction of synthesis. Nothing has been found which indicates that the autolytic cell disintegration in cancer tissue can be directly connected with the enzymic action of cathepsin. It is much more likely that arginase is connected with this autolysis.

Experimental.

Catheptic activity of liver, muscle and tumour. Immediately after extirpation, the organs to be examined for catheptic activity were cut up with scissors, frozen in liquid nitrogen and then crushed with a hammer. This pulverised material was suspended (1 : 10) in 90 % glycerol. For activation, 2·0 ml. of neutral cysteine HCl solution (containing 20 mg. of cysteine, HCl) were added to 7·0 ml. of the well mixed enzyme tissue suspension and allowed to stand for 30 minutes at 30°, at p_H 7. 5·0 ml. of 8 % gelatin (Kahlbaum "gold label") and 2·0 ml. of 1 *M* acetate buffer (p_H 4) were then added, and the volume was made up to 25 ml. After incubation at 30° for 24 hours, the increase in amino-nitrogen was determined by the Van Slyke method.

Table I shows the changes in cathepsin concentration, during the ageing of the tumour, in muscle, liver and tumour tissue of rats into which cancer had

Table I. *Catheptic activity of liver, muscle and tumour of cancerous animals.*

	Sarcoma					Liver		Muscle	
Rat	P.	F.	N.	Initial activity	Full activity	Initial activity	Full activity	Initial activity	Full activity
1	85	15	—	1·0	2·90	—	—	—	—
2	65	35	—	1·05	2·80	0·84	1·80	0·12	0·35
3	65	30	5	0·70	2·40	—	—	—	—
4	50	45	5	0·64	2·0	0·32	1·20	0·10	0·25
5	40	45	15	0·40	1·70	1·0	1·50	0·08	0·33
6	40	40	20	0·56	1·90	0·50	1·70	—	—
7	35	55	15	0·52	1·80	1·31	2·80	0·10	0·30
8	35	65	—	0·80	2·50	0·70	1·65	0·12	0·20
9	30	70	—	0·16	1·00	0·30	1·31	—	—
10	30	50	20	0·90	1·56	0·42	1·31	0·09	0·25
11	30	50	20	0·56	1·44	1·20	2·32	0·10	0·28
12	25	50	25	0·56	1·00	0·84	2·10	—	—
13	25	25	50	0·65	1·65	1·68	2·52	0·10	0·25
14	25	20	55	0·72	1·00	2·80	3·36	0·15	0·33
15	20	60	20	0·24	0·75	1·68	2·10	0·09	0·28

successfully been transplanted. The tumour used was a rapidly growing sarcoma, Philadelphia #1, the histological description of which may be found in the paper by Waldschmidt-Leitz, McDonald and co-workers [1933]. The results are given for 10 ml. of the reaction mixture (corresponding with 2·8 ml. of enzyme suspension) and indicate the increase of NH_2 expressed as ml. of 0·05 *N* KOH. P. indicates parenchymal tissue; F. fibrous tissue; N. necrotic portions of the tumours, stated in percentages.

Table II shows the catheptic activity of liver and muscle of healthy normal rats and of cancer-resistant rats (in which transplants failed to grow). The experimental details are identical with those described for Table I.

Table II. *Catheptic activity of liver and muscle of healthy and cancer-resistant rats.*

Healthy rats (average body weight—90 g.)				Cancer-resistant rats (average body weight—85 g.)			
Liver		Muscle		Liver		Muscle	
Initial activity	Full activity	Initial activity	Full activity	Initial activity	Full activity	Initial activity	Full activity
1·05	1·75	0·05	0·09	1·75	2·62	0·10	0·30
0·56	1·68	0·02	0·05	1·12	2·27	0·09	0·15
0·25	1·45	0·00	0·00	1·20	2·30	0·08	0·20
0·56	1·38	0·00	0·00	1·80	2·90	0·10	0·25
0·56	1·12	0·00	0·00	1·50	3·10	0·15	0·30
0·32	1·30	0·00	0·05	1·00	2·50	0·08	0·12
0·65	1·50	0·00	0·00	1·20	2·50	0·05	0·15

Catheptic activity of histologically homogeneous parts of cancer tissue. The experimental material in this series of experiments was obtained from a transplantable rat sarcoma, Philadelphia #1, and a transplantable rat carcinoma, Walker #256. The latter is a carcinoma tending to be necrotic in the early stages. The histological description of both tumours is given by Waldschmidt-Leitz, McDonald and co-workers [1933].

The experimental material of the desired histological structure was first prepared macroscopically and then microscopically by staining a section with haematoxylin-phloxin. The further procedure of the experiment was the same as that described under Table I.

Table III. *Catheptic activity of histologically homogeneous parts of cancer tissue.*

Cancer		Parenchymal tissue			Fibrous tissue			Necrotic tissue		
Strain	Age of tumour (weeks)	p_H of tissue	Initial activity	Full activity	p_H of tissue	Initial activity	Full activity	p_H of tissue	Initial activity	Full activity
Phila. #1	6	6·3	0·5	1·4	6·4	0·28	0·60	—	—	—
,,	4	6·0	0·7	1·8	6·5	0·20	0·90	—	—	—
,,	3	6·0	1·0	2·5	—	—	—	—	—	—
Walker #256	4	6·3	0·40	1·10	—	—	—	6·9	0·00	0·20
,,	3	6·0	0·50	1·50	—	—	—	7·0	0·00	0·10
,,	3	6·2	0·70	1·90	—	—	—	7·0	0·00	0·09
Arginase activity*										
Fe″ Cysteine				4·0						17·6

* See Waldschmidt-Leitz, McDonald and co-workers [1933].

SUMMARY.

1. Untreated laboratory albino rats show a significant and uniform cathepsin content in liver, while the muscular system is free from this enzyme or has it in insignificant amount.

2. In rats into which cancers have successfully become transplanted there is a significant increase of the cathepsin concentration in the liver and coincidentally a large amount of cathepsin now appears in the muscle. The increase of cathepsin in liver and its appearance in muscle do not run parallel with the decline of the cathepsin concentration in the ageing cancer tissue (increase of necrosis), but are irregular and indicate an unsettled enzymic metabolism.

3. Rats into which efforts to transplant cancer have failed prove to have a particularly high concentration of cathepsin in liver and a significant concentration of cathepsin in muscle. This contrasts with the findings in normal rats in which no effort to transplant has been made.

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CCL. THE DISTRIBUTION OF PEROXIDASE IN ANIMAL TISSUES.

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The enzyme peroxidase has commonly been believed to be present in all animal tissues. It appears that no real evidence to this effect has yet been presented and the assumption seems to be based on early crude experiments and on the results of histological studies. The latter depend on the colours produced in fixed sections by strong hydrogen peroxide with benzidine, *p*-phenylenediamine and similar reagents. Such colours will, however, be given by any haematin compound, as for instance haemoglobin, in acid solution with strong H_2O_2. We would like to distinguish between the enzyme and other types of catalyst which promote oxidations by H_2O_2. The enzyme shows its full activity in neutral solution and at concentrations of H_2O_2 which are possible within the organism. Under these conditions the thermostable catalysts (see below) have less than one-ten-thousandth of the activity of a concentrated enzyme preparation.

The formation of H_2O_2 as a stage in the oxidation of organic substances is an integral part of Wieland's theory, and its formation has been demonstrated in various plant, bacterial and animal systems, as for instance in oxidations catalysed by the yellow ferment of Warburg. Though Elliott [1932, 1, 2] showed that peroxidase catalysed the direct oxidation of none of the common metabolites except tryptophan and phenolic compounds, he did not infer that the enzyme could have no general importance, rather suggesting that the metabolites may also need previous activation as in the cytochrome-oxidase system of Keilin.

We therefore felt it desirable to obtain an estimate of the peroxidase activity in the various tissues of normal animals and in tumours.

It seemed best to use for the estimation of peroxidase a method in which the conditions approached as closely as possible to those of Willstätter and Stoll [1918] in order that we could express results in terms of the unit of concentration "P.Z." defined by him and since used by various other workers.

In this method, as indeed in any method, it is necessary to have a definite concentration of hydrogen peroxide in the reaction mixture. Most tissues contain catalase which rapidly decomposes H_2O_2 and numerous methods were tried to remove catalase without damaging the peroxidase content. All such methods failed, and therefore a method was devised for compensating for the H_2O_2 decomposed.

Most of the experiments on the removal of catalase were tried on pig's spleen. Spleen was found to be an organ with comparatively high peroxidase content and though it contains a fair amount of catalase the amount was not so great as to vitiate entirely estimations of peroxidase activity; we were thus able to attempt to remove the catalase and test the effect of the treatment on the remaining peroxidase content.

We had hoped to be able to extract the peroxidase completely from the tissue, and to be able then to work with a clear centrifuged extract. However, though the tissue was minced to a fine paste in a Latapie mincer and then ground with sand and extracted with large volumes of water it was found that only about half the peroxidase activity went into solution, the remainder staying with the tissue debris. For instance, 9 g. of pig's spleen minced and ground with sand were shaken for an hour with 1800 ml. of distilled water. On taking 100 ml. of the suspension (500 mg. tissue) and making up to 500 ml. the usual estimation method gave 4·7 mg. purpurogallin. After centrifuging the suspension, 200 ml. of the centrifugate gave only 4·74 mg. purpurogallin. These amounts correspond respectively to P.Z. $9{\cdot}4 \times 10^{-3}$ and $4{\cdot}7 \times 10^{-3}$ on the wet weight of tissue. Neither alkaline phosphate solution nor 50 % glycerol removed the activity completely from the tissue. Extraction with glycerol, however, seems to bring out the peroxidase activity better than pure water, since the glycerol suspensions have higher activities than uncentrifuged aqueous suspensions. With a completely soluble milk peroxidase preparation the presence of glycerol was without effect, so the action of glycerol may depend on its viscosity maintaining a better dispersion of tissue particles.

This division of peroxidase into a dissolved and an undissolved fraction has previously been remarked by Willstätter and Pollinger [1928] in horseradish tissue, and with various other enzymes, and the terms desmo- and lyo-enzymes have been introduced to distinguish the two fractions.

Attempts were made to liberate the undissolved enzyme by drying the tissue and extracting the powdered residue. The minced tissue was dried with ether or acetone, or *in vacuo* over sulphuric acid. The treatment resulted always in the loss of about half the activity, and of the remainder only half could be got into clear solution. (No activity was found in the acetone or ether extracts.) Treatment of the tissue with alcohol always resulted in complete destruction of the peroxidase.

Undried tissue suspended in 50 % glycerol gave P.Z. $41{\cdot}0 \times 10^{-3}$.

The same after centrifuging gave P.Z. $29{\cdot}2 \times 10^{-3}$.

Acetone-dried tissue suspended in 50 % glycerol after centrifuging gave P.Z. $16{\cdot}9 \times 10^{-3}$.

In studying the possibility of removing catalase, clear centrifuged extracts were used. It is well known that alumina adsorbs catalase out of aqueous solution, while horseradish peroxidase is adsorbed on alumina from 50 % alcohol and eluted by pure water, and this difference seemed to offer a ready method for separating catalase. When, however, centrifuged aqueous extracts of spleen were treated with various amounts of Willstätter's alumina A or alumina C, the amount of adsorbent which would remove the catalase also removed the peroxidase practically completely.

Similarly calcium phosphate adsorbed a considerable amount of peroxidase as well as catalase. It seems possible that this adsorption of peroxidase on alumina, under conditions under which purified plant peroxidase is not adsorbed, may be due to the association of the enzyme with adsorbable proteins in the crude extract. Similarly the inactivation by alcohol may be due to a precipitation of the enzyme so occluded in denatured protein that it is unable to catalyse reaction in solution.

In the hope of liberating all the peroxidase in soluble form, attempts were made to digest away the proteins (by trypsin and papain) but these efforts failed owing to the rapid inactivation of peroxidase at temperatures of 25–35°.

Alcohol is without effect on the peroxidase of horseradish and is used for precipitating and drying preparations from this source. Alumina does not adsorb peroxidase from a crude aqueous extract of horse-radish; and drying such an extract *in vacuo*, and treating the dried solid with alcohol, do not affect its activity. It is clear that the proteins associated with the enzyme from different sources are very different.

Method of estimation.

The basis of the method was that of Willstätter and Stoll [1918]. This method consists simply in adding 10 ml. of 0·5 % H_2O_2 to two litres of water at 20° containing 5 g. of pure pyrogallol and a measured amount of the enzyme. The amount of enzyme is so chosen that 15–20 mg. of purpurogallin are formed in 5 minutes. At the end of 5 minutes the reaction is stopped with sulphuric acid, and the purpurogallin extracted with ether and estimated colorimetrically. Elliott and Keilin [1934] found the estimation satisfactory and more convenient using one fourth of the above quantities.

Since it seemed impossible to obtain the whole of the peroxidase in clear solution or free from catalase we used a fine suspension of the tissue in 50 % glycerol. During the whole five minutes of the reaction, under vigorous mechanical stirring, a solution of H_2O_2 of the strength calculated to maintain the initial H_2O_2 concentration was added.

The catalase activity of the suspension was first determined and the constant k of the unimolecular decomposition of H_2O_2 obtained from the equation $k=\frac{2\cdot3}{t}\log_{10}\frac{a}{(a-x)}$ where t = time in minutes, a and $(a-x)$ are the concentrations of H_2O_2 at the beginning and at time t respectively. The velocity, V, of decomposition of H_2O_2 per minute by the catalase is then given by $V=kc$ where c is the concentration of H_2O_2 (2·5 mg./100 ml.) required in the experiment.

The solution added per minute therefore should contain V *plus* the required experimental concentration c, that is to say the concentration C of H_2O_2 in the added solution is given by

$$C=c+V \text{ or } C=c\,(1+k).$$

In practice, owing to complex variations from a simple law with tissue suspensions, this calculation served only as a rough guide, and the actual amount of H_2O_2 necessary to add was usually somewhat greater.

It was necessary to avoid using too strong a solution of H_2O_2 lest local concentration of H_2O_2 might harm the peroxidase before it became well mixed; we therefore started the reaction in a volume of 250 ml. containing the proper concentrations of H_2O_2 and pyrogallol and during the five minutes 500 ml. of a solution containing 1·25 g. of pyrogallol and H_2O_2 of the necessary strength were added at the rate of 100 ml. per minute. Thus the mean volume of the reaction mixture throughout the experiment is 500 ml., the pyrogallol concentration remains the same and the H_2O_2 concentration is kept at the right level. To maintain a p_H of 6 during the reaction, the mixture contained $M/200$ phosphate buffer.

The phosphate used had to be carefully freed from traces of copper and this was done by treating KH_2PO_4 solution with H_2S, filtering, boiling off excess of H_2S and recrystallising twice from water. The buffer was then made up to p_H 6 with pure NaOH solution. Untreated phosphate from Merck and Kahlbaum both form a yellow colour with pyrogallol and H_2O_2 quite rapidly. That this is due to traces of copper is suggested by the fact that HCN and H_2S inhibit the action while pyrophosphate does not. A trace of copper in the phosphate could indeed be detected.

spectroscopically. Neither ferrous nor ferric iron in small amount would produce this reaction, while 0·01 mg. of copper sulphate in 500 ml. solution caused colour to be developed in a few minutes.

For the above reasons redistilled water was used throughout the work.

Since the experimental solutions contain a large amount of protein which causes obstinate emulsification in the ether extraction, it is necessary to stop the reaction with an acid protein precipitant, rather than H_2SO_4, and to filter before extraction.

Pyrogallol is easily oxidised to give a deep red colour, apparently by ether peroxides under the catalysis of iron or other complexes, and this is especially noticeable with tissue extracts and can seriously interfere with the colorimetric estimation of purpurogallin. To avoid this, ether of the best quality should be used; good salicylsulphonic acid dissolved in glass-distilled water is used as protein precipitant. For the filtration only an acid-washed paper such as Whatman No. 40 should be used.

Tissue peroxidase rapidly loses its activity in solution on standing, but it was found possible to keep organs moistened with Ringer solution or minced tissue in covered flasks for one or two days in the ice-box without appreciable loss.

The following is a detailed example of the procedure with a specimen of rat-liver.

The animal was killed by a blow on the head followed by bleeding from the neck. A weighed sample of the liver was dried in an oven at 100–110° to determine the dry weight/wet weight ratio. Then 4·4 g. of the liver were thoroughly ground with about 0·5 g. sand and shaken gently in a shaking machine for an hour with 80 times its weight of 50 % glycerol. To determine the catalase activity, 3 ml. of the suspension were mixed with 20 ml. of $M/20$ phosphate buffer p_H 6·0 and 0·5 g. of pyrogallol[1] and made up to 200 ml. with redistilled water. To this 1·4 ml. (7·0 mg.) H_2O_2 solution were added and 10 ml. samples were withdrawn at the time intervals 0, 2, 4 and 6 minutes and run into 50 ml. flasks containing 5 ml. of 10 N H_2SO_4. To each flask 4 ml. of 5 % KI were added, and after five minutes the iodine liberated was titrated with 0·005 N thiosulphate. This gave the amount of H_2O_2 remaining after the various time intervals and from this the velocity constant of decomposition was calculated.

Time	Titration (ml.)	$\frac{a}{a-x}$	$k=\frac{2\cdot3}{t}\log_{10}\frac{a}{a-x}$
0	2·98	—	—
2	1·58	1·84	0·31
4	0·89	3·27	0·30
6	0·50	5·82	0·29

The constant for 3 ml. of suspension being 0·3, that for 200 ml. (the amount necessary for the peroxidase estimation) is 20. The amount of H_2O_2 necessary to add per minute to maintain a concentration of 2·5 mg./100 ml. is given by the expression $c\,(1+k)$ and is therefore $2\cdot5\,(1+20)=52\cdot5$ mg. or 263 mg. for 5 minutes.

Of the suspension 200 ml. were made up to 250 ml. with redistilled water containing 0·625 g. pyrogallol and 25 ml. of buffer. This mixture was put in a precipitating jar, adjusted to 20° and vigorously stirred with a mechanical stirrer throughout the experimental period. At zero time 1·25 ml. of 0·5 % H_2O_2 were

[1] The presence of pyrogallol depresses the activity of catalase considerably. It is therefore necessary to have its concentration in the catalase determination the same as in the peroxidase estimation.

added. At the same time 500 ml. of a solution containing 50 ml. buffer of p_H 6, 1·25 g. pyrogallol and 320 mg. of H_2O_2, were run in steadily at the rate of 100 ml. per minute. (This amount of H_2O_2 is more than that theoretically required but was found actually to be necessary.) A constant rate of addition of this solution was obtained by letting the solution run in from a small funnel which was kept full, the stem of which had been adjusted to give the correct flow.

At the end of five minutes 35 ml. of 44 % sulphosalicylic acid were added to stop the reaction and precipitate the proteins. The fluid was filtered through Whatman No. 40 paper and extracted three times with a total of 200 ml. of good ether. The purpurogallin in the ether was estimated colorimetrically against a standard solution of purpurogallin in ether. In this case 2·52 mg. were formed,

$$\text{P.Z.} = \frac{\text{mg. purpurogallin}}{\text{mg. tissue}} = \frac{2{\cdot}52}{2500} = 1{\cdot}08 \times 10^{-3} \text{ on wet weight.}$$

The ratio wet weight/dry weight was found to be 3·08 whence

$$\text{P.Z.} = 3{\cdot}3 \times 10^{-3}$$

on the dry weight.

The H_2O_2 was estimated in a sample of the filtrate before extracting with ether and 2·1 mg./100 ml. were found present showing that the concentration of H_2O_2 has been successfully maintained near the initial 2·5 mg./100 ml.

As checks on the method the following experiments were tried.

First a preparation of milk-peroxidase [Elliott, 1932, 1] was mixed with a preparation of liver-catalase prepared according to Morgulis [1926].

The activity of the milk-peroxidase was determined in the ordinary way and found to be P.Z. 0·98.

Of the peroxidase 4·1 mg. were mixed with 8·0 mg. of the catalase preparation. The catalase activity of the mixture at 20° and in the presence of pyrogallol was found to be Kat. f. 160 on the dry weight of the preparation and on carrying out the peroxidase estimation on the mixture according to the method above described, the P.Z. on the dry peroxidase was found to be 0·96.

In a suspension of rat-liver tissue the catalase and peroxidase were estimated. Using 40 ml. of the suspension, 0·5 mg. of purpurogallin was formed in the estimation. The peroxidase estimation was then repeated, but to the suspension 4·0 mg. of milk-peroxidase of P.Z. 0·8 were added. Then 3·5 mg. of purpurogallin were formed, which means 3·0 mg. (3·5–0·5) of purpurogallin due to the added peroxidase. As 4·0 mg. of peroxidase give 3·2 mg. of purpurogallin, the result is in fair agreement with that expected.

Finally a suspension of liver tissue was allowed to stand for several days. The suspension then contained no peroxidase but contained catalase. On adding 3·3 mg. of milk-peroxidase of P.Z. 1·27 and carrying out an ordinary estimation, 4·3 mg. of purpurogallin were formed. This is again in good agreement with the 4·2 mg. of purpurogallin expected and shows that reducing substances and other materials in the suspension do not seriously interfere with the estimation.

Results.

The distribution of catalase has been previously studied (see for instance Hawk [1911]) but we show here some of our own determinations of the catalase content of the various tissues so that the relative distributions of the two enzymes may be compared. These determinations are not directly comparable with catalase determinations made according to Euler and Jacobsen [1926] since we worked

at 20° instead of 0° and in presence of pyrogallol which has a strong inhibitory effect, nevertheless we have used their term for catalase activity, namely

$$\text{Kat. f.} = \frac{k\ (\text{constant of unimolecular reaction})}{\text{g. dry weight}}.$$

With all tissues tried it was found that after boiling the suspension the peroxidase activity was negligible.

Table I.

Figures in brackets are P.Z. × 10^3 on wet weight.

		Peroxidase activity P.Z. × 10^3 on dry weight	Catalase activity (in presence of pyrogallol see above) Kat. f.
Rabbit	spleen	70 (17), 61, 113	4·6, 4·5
	lung	42 (8·4)	Trace
	kidney cortex	4·6 (1·1), 3·7	23·0
	liver	7·3, 2·4, 8·7 (2·5)	3·6, 7·2
	brain	Trace	None
	adrenal	None	3·5
	muscle	Trace	None
	skin	None	None
	red bone marrow	7·5 (5·7)	2·0
	serum	None	—
Rat	spleen	105 (24), 70, 100*, 221*	3·4, 5·5, 2·9*, 9·0*
	lung	83 (17), 31	2·2, 1·9
	whole kidney	2, 9·5 (1·9), 4·0*, 3·5*	8·8, 16·7, 5·6*, 10·3*
	liver	3·3, 4·1, 4·6* (1·4), 2·8*	26·5, 42·1, 17·0*, 17·3*
	mammary gland	None	None
	sarcoma (Philadelphia No. 1)	1·8 (0·3), 1·3	Trace
	carcinoma (Walker No. 256)	Trace	Trace
Chick embryo (8 day)		Trace	Trace
Horse melanotic sarcoma		7·0	2·0
Cow's milk		(22)	—
Cow's colostrum		9·0 (3·2)	—

* These estimations were made on healthy organs of tumour-bearing animals.

Remembering that Willstätter and Pollinger [1923] have obtained preparations with activity up to P.Z. 4700, it is seen from Table I that in no tissue tried is there any remarkably high peroxidase activity. Though the activity in any organ of different individuals varies considerably, the spleen appears to have the highest peroxidase content with a P.Z. of about 0·1, and lung the next highest. Kidney and liver follow but are much less active, while brain and muscle are practically inactive. In two types of malignant tumour activity seems absent while in a melanotic sarcoma there was some peroxidase.

The following consideration is of some interest. The activity of rabbit spleen is about P.Z. 70×10^{-3}, *i.e.* 1 mg. dry weight of tissue produces about 0·07 mg. purpurogallin in 5 minutes. From the formula, $C_{11}H_5O_8$, of purpurogallin it is calculated that $5{\cdot}4 \times 10^{-3}$ mg. of H_2O_2 must be activated to oxidise pyrogallol to this amount of purpurogallin. If tissue oxidations follow the Wieland formula completely ($AH_2 + O_2 = A + H_2O_2$) then for 22400 ml. of O_2 uptake 34 mg. H_2O_2 should be formed. The oxygen uptake rate of rabbit spleen is roughly 10 ml. per mg. dry weight per hour. If all this oxygen formed H_2O_2 there would be produced 34/22400 mg. H_2O_2 per hour, or in 5 minutes, $1{\cdot}25 \times 10^{-3}$ mg. Hence it appears that there is more than enough peroxidase activity in spleen to use all H_2O_2 formed supposing all oxygen uptake followed the Wieland mechanism. In liver

and kidney there would seem to be rather less than enough activity (one-third of that required) to account for all O_2 uptake proceeding through H_2O_2. However, it must be remembered that there is no reason to suppose that substances in the cell, possibly previously activated by other enzymes, would be oxidised by H_2O_2 and peroxidase at the same rate as is pyrogallol in our experimental conditions. The above consideration merely shows that in spite of low activity, such a mechanism is not disproved in the case of some organs. Nevertheless it seems to us most unlikely that peroxidase plays any large part in animal tissue oxidations, especially in view of the perfusion experiments in the next section.

"True" peroxidase and haemoglobin derivatives.

Willstätter and Pollinger [1928] found that oxyhaemoglobin from various animals had a peroxidase activity of P.Z. 0·06 to 0·13 under the normal estimation conditions. This value is considerably higher than that found in many organs, so it seemed possible that the activity found by us was due to haemoglobin in the tissues. We have attempted to check this possibility by perfusing organs to render them as free as possible from blood. Rabbits were anaesthetised with amytal, and warmed Ringer-Locke solution containing heparin was run in through the jugular vein while the animal was bled from the carotid artery, and the heart was massaged when it ceased beating. Finally the organs were separately perfused by syringing Ringer solution into main vessels. After this procedure the lung became almost pure white, the liver and kidney very much paler, but the spleen could not be very successfully perfused. Stained sections of the organs were prepared and showed that the tissues had been washed free of red blood corpuscles, except in the spleen where some corpuscles still remained. The experiment was tried on three animals and the following results obtained (Table II).

Table II. *Peroxidase activity of perfused rabbit organs.*

	P.Z. $\times 10^3$		
Spleen	—	29	88
Lung	50	52	—
Kidney	—	Trace	Trace
Liver	—	Trace	Trace

While the activity in kidney and liver appears to be almost completely removed, the activity of lung seems unaffected even though perfusion must have removed practically all haemoglobin. All the activity of spleen could certainly not be accounted for by haemoglobin since the activity of this tissue is of the same order as that of the pure pigment.

Since other haematin derivatives are known to be present in the cell a survey of the activity of various haematin compounds was made. After the experimental 5 minutes the protein was precipitated with salicylsulphonic acid, as in other experiments. The following summarises our findings.

Alkaline haematin. Haemin crystals were dissolved in a minimum amount of dilute alkali and the activity of the solution estimated in the usual way at p_H 6; found P.Z. 125×10^{-3} on the haematin, or P.Z. 1·57 on the haematin Fe.

Pyridine haemochromogen. Some of the above solution was reduced with a trace of sodium hydrosulphite[1] and pyridine added; found P.Z. 393×10^{-3} on the haemochromogen, or P.Z. 4·5 on the haematin Fe.

Nicotine haemochromogen. Nicotine was added instead of pyridine; found P.Z. 232×10^{-3} on haemochromogen, or P.Z. 4·2 on the haematin Fe.

[1] The hydrosulphite did not affect the H_2O_2 concentration appreciably.

Oxyhaemoglobin. Freshly prepared crystalline oxyhaemoglobin was kindly given us by Dr W. C. Stadie, found P.Z. 53×10^{-3} on the haemoglobin, or P.Z. 16 on the haemoglobin Fe.

CO-haemoglobin. Carbon monoxide was prepared from formic acid and H_2SO_4, washed through NaOH solution and water and bubbled through the haemoglobin solution before and during the experiment; found P.Z. 66×10^{-3}. There is thus no inhibition by CO of the catalytic effect of haemoglobin. Elliott and Sutter [1932] found that CO does not affect other peroxidase preparations.

Methaemoglobin. Haemoglobin solution was treated with potassium ferricyanide and the excess salt dialysed away in the ice-box; found P.Z. 95×10^{-3}.

Kathaemoglobin. Methaemoglobin solution was shaken with a little chloroform; found P.Z. 118×10^{-3}.

Haemochromogen. Haemoglobin solution was treated with alkali and warmed, then sodium hydrosulphite was added; found P.Z. 57×10^{-3} on the haemoglobin.

Acid haematin. Haemoglobin solution was treated with acetic acid and boiled, cooled and neutralised with NaOH. Though the pigment remained in fine suspension all activity was destroyed.

The activity of oxyhaemoglobin on standing in the ice-box for a week or more increases somewhat, probably owing to slow denaturation with the formation of kathaemoglobin. The following results were obtained with preparations from older haemoglobin; oxyhaemoglobin P.Z. 67×10^{-3}, methaemoglobin P.Z. 90×10^{-3}, kathaemoglobin 104×10^{-3}.

A small amount of concentrated cytochrome C solution kindly given to one of us by Prof. D. Keilin was found to have negligible peroxidase activity ($<$ P.Z. 20×10^{-3}) according to our estimation method.

Discussion.

There is in horseradish and other plants a peroxidase enzyme which it is possible to concentrate in very active form. A similar enzyme appears to be present in milk, since Elliott [1932, 1] was able to obtain from milk a product with P.Z. as high as 4·7 although the preparation must have consisted largely of inactive lactalbumin. To what extent such an enzyme accounts for the small activity of animal tissues is difficult to decide. The criterion of thermolability hardly applies, since though activity in tissues is destroyed by boiling, a haemoglobin solution on boiling will coagulate and lose its activity. Even without coagulation, heat can cause inactivation as in the case of the acid haematin solution mentioned above. Of all the haematin compounds tested, only the pyridine and nicotine haemochromogens have activities higher than the haemoglobin derivatives (though the activity per unit of iron is actually lower), and these are not probable cell constituents. Lung and spleen do not consist almost entirely of haemoglobin substances, so it seems likely that there is in these organs some "enzyme" peroxidase. The activity of kidney and liver is lost on perfusion and so may be all due to haemoglobin. The "enzyme" peroxidase may itself be a special haematin compound though the work of Elliott and Keilin [1934] makes this doubtful.

Summary.

1. Tissue peroxidase occurs in two fractions, one soluble in water and another associated with insoluble material.

2. Various attempts to remove catalase without affecting the peroxidase in solutions and suspensions failed.

3. A method for estimating peroxidase in the presence of catalase is described.

4. The peroxidase activity of various tissues has been determined. It is highest in the spleen and lung; liver and kidney contain a little. In most other tissues it is negligible.

5. The peroxidase activity of various haematin and haemoglobin derivatives has been estimated.

6. Perfusion removes the activity from liver and kidney; the activity therefore appears to be due to haemoglobin in blood corpuscles. In spleen and lung it is likely that there is some true peroxidase besides the activity due to haemoglobin and derivatives. It does not seem probable that peroxidase plays an important part in oxidations in any organ except possibly in lung and spleen.

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CCLI. THE METABOLISM OF LACTIC AND PYRUVIC ACIDS IN NORMAL AND TUMOUR TISSUE.

I. METHODS AND RESULTS WITH KIDNEY CORTEX.

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The formation and breakdown of lactic acid have been very extensively studied in muscle tissue and bacteria; in animal tissues other than muscle the metabolism of lactic and pyruvic acids has been investigated chiefly by feeding, injection and perfusion experiments on whole animals and organs.

The introduction by Warburg of the surviving tissue slice technique with manometric measurements has given us a considerably more precise means for studying local intermediary metabolic processes, and lately the usefulness of such methods has been demonstrated by Krebs and Dickens and their collaborators and others.

The apparatus recently developed by Dixon and Keilin [1933] has provided a means whereby the oxygen uptake, respiratory quotient and acid formation or disappearance can be measured simultaneously on the same sample of tissue in the presence of a bicarbonate-containing medium. The work described in this series should demonstrate the utility of this apparatus and, we hope, throw considerable light on the activity of the various tissues in metabolising lactic and pyruvic acids, including the intermediate stages in the breakdown of these substances. Combined with the manometric experiments, chemical estimations are carried out on the manometer contents to determine formation or disappearance of lactic acid, pyruvic acid and glycogen.

In this paper we describe the details of our methods and the results obtained with kidney cortical tissue, chiefly of the rabbit. Though this tissue does not form or metabolise lactic acid to any great extent, it appears [Krebs, 1933] to be the chief seat of the oxidative deamination of amino-acids and would be expected therefore to have a high power for dealing with keto-acids. Later papers will deal with other tissues amenable to the method in the hope that light may be thrown on differences of behaviour in tissues such as tumour, wherein lactic acid is rapidly formed, brain and diaphragm which have the capacity for forming it anaerobically and liver wherein glycogen appears to be produced from lactic and pyruvic acids [see *e.g.* Meyerhof *et al.*, 1926]. The question of the metabolism of keto-acids other than pyruvic acid may be studied later.

I. Definition of terms.

Throughout this work we use the terms proposed by Warburg for oxygen uptake and CO_2 output, namely,

$-Q_{O_2}=\mu$l. O_2 taken up per mg. dry weight of tissue per hour.
$Q_{CO_2}=\mu$l. CO_2 given out per mg. dry weight of tissue per hour.
(All gases measured at N.T.P.)

In place of his term for "glycolysis" we propose to introduce two more precise terms, namely,

$Q_A=\mu$l. CO_2 given out by any acid formation per mg. dry weight of tissue per hour.
$Q_{LA}=\mu$l. CO_2 given out by lactic acid formation per mg. dry weight of tissue per hour.

The manometric results will thus give Q_A, the acid formation (or, when negative, disappearance). We suggest the term "acidogenesis" for this quantity. The results of chemical estimation of lactic acid will give Q_{LA} (1 mg. lactic acid = 249 μl. CO_2). Q_A will thus represent the algebraic sum of the formation and removal of lactic acid, pyruvic acid and any other acids, or their neutralisation by, for instance, NH_3 production.

In order to reduce all other quantities measured to equivalent terms, two more terms, for pyruvic acid and for glycogen, will be used. That for pyruvic acid will be,

$Q_{Pyr}=\mu$l. CO_2 liberated by the production of pyruvic acid, per mg. dry weight of tissue per hour.

This quantity will be obtained from chemical estimation of pyruvic acid (1 mg. = 255 μl. CO_2).

Though changes in amount of glycogen do not affect the manometric readings, for uniformity and convenience in discussing results the following term will be used,

$$Q_{Ps}=\frac{\text{mg. of glycogen formed}\times 277}{\text{mg. dry weight}\times\text{hours}};$$

(Ps = Polysaccharide)

when glycogen is estimated as glucose, the factor becomes 249 instead of 277. This factor is arrived at by considering the glycogen as $2n$ ($C_3H_5O_{2\cdot5}$), and regarding it as a 3 carbon compound similar to the lactic or pyruvic acid.

As a general term to describe the units used thus in manometric work, we propose the term M.E., *i.e.* Manometric Equivalent.

II. Methods.

Apparatus. The manometers used were of the Dixon and Keilin [1933] type and fulfilled the specifications made by them in every respect. The dimensions of the manometers were determined as described by Dixon and Keilin and the constants for O_2 and CO_2 calculated from their formula. Three sets of constants, however, were calculated for use according to the contents of the vessels. Constants A were for use when only 3 ml. of fluid were present; for B, 200 μl. were deducted from the gas volume to allow for hanging cups (see below) and about 200 μl. were added to the fluid volume and deducted from the gas volume to allow for tissue present, and constants C were for cases when tissue, but no hanging cups, was present. Constants for readings taken at 10° were also calculated for use in certain experiments.

In the case of rapidly metabolising substrates it was necessary to have the substrate, dissolved in the proper medium, in a small cup hooked by a short platinum wire to the alkali tube as described by Keilin [1929]. These, present in both sides, could then be shaken down after passing gas, equilibrating and turning acid into the left cup. These "danglers" were large enough to contain 0·3 ml. of liquid and were made with oval cross-section. This shape made them easier to introduce with forceps, and as they could not roll about there was less danger of damage to the tissues. Experiments with coloured liquid showed that the contents very rapidly washed out uniformly into the rest of the medium. To ensure their dropping easily the wire hook was bent so that they only just hung, and the paper in the alkali-tube was bent back away from the hook. Unequal measurement into the two vessels of the bicarbonate-containing medium can produce large errors. For this purpose, therefore, a well-cleaned 5 ml. burette graduated in 0·01 ml. and connected directly to the tonometer containing the medium was used.

For the displacement upwards of the strong alkali, glass rods and not mercury were always used. With these there is no danger of splashing out mercury when shaking down the danglers and there is the advantage that, providing excess grease has been removed from the inside tube, they never stick; and the fact that they have gone down properly is known since the alkali tap cannot be turned again. The rods were of pyrex glass about 26 mm. long and 5 mm. in diameter and interchangeable, all being of equal volume.

It is essential that after filling the strong alkali into the well in the bottom stopper, the latter should be turned until it is only just closed off. Otherwise changes of temperature and pressure cause globules of alkali to work up through the grease into the vessel giving premature absorption of CO_2. Elastic bands can be strained on so that the stop-cock remains in this position.

A smooth rubber-containing grease was used for the bottom stopper and was also found necessary for the bored side-stoppers unless they were very perfectly ground. Anhydrous lanoline was used for all other joints and stop-cocks. Care should be taken that no grease gets on the edge of the side-bulb as otherwise the acid may fail to flow into the vessel when the bulb is turned. The stop-cocks on the manometer itself must be well cleared so that a proper equilibrium may be reached between the two vessels after passing gas and before starting the experimental period. The readings for h_1 and h_2 were not taken until equilibrium was reached. For h_1, that is after turning acid into the experimental vessel, about 25 minutes were allowed. The absorption of CO_2 is rather slower than mentioned in Dixon and Keilin's paper and it is rarely safe to take the final reading of h_2 until one and a half to two hours after introduction of the alkali. At the end of the experiment the manometer taps should be opened without moving the apparatus, and after about ten minutes a zero reading taken to see whether any correction is to be applied to h_1 and h_2.

The vessels *etc.* should be cleaned soon after using so that the time of contact of some parts with strong alkali is minimised. For cleaning, the following series seemed best: wipe with paper, rinse in xylene, alcohol, strong chromic acid for 30 minutes, cold and hot tap-water, distilled water. If, after the chromic acid, black patches remain, these are best removed with a soapy pipe cleaner and cold water.

The bath employed was of the ordinary type used with the Barcroft differential manometer; it was of such a size that three simple manometers or two Dixon-Keilin apparatus could be worked with on either side of the stirrer. The apparatus for passing gas into the vessels is shown in Fig. 1. It is similar to that

used by Dixon and Keilin but includes a system for purifying and leading a 95 % N_2–5 % CO_2 gas mixture for anaerobic experiments. The two wash bottles for saturating the gas with water vapour stand in the water-bath at 37°. The right-hand distributor and wash bottle are used only for anaerobic experiments and when not in use are kept closed to prevent oxygen from dissolving in the water.

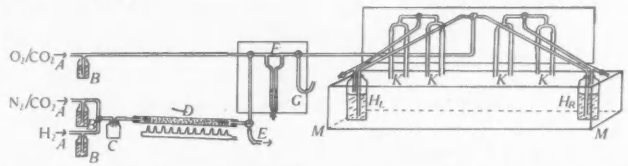


Fig. 1. Diagram of gas-leading system. *A*, gases from cylinders; *B*, mercury safety bottles to release excess pressure; *C*, trap to prevent mercury reaching copper; *D*, tube containing reduced copper wire, heated to dull redness to remove oxygen in anaerobic experiments; *E*, escape for H_2 when reducing copper oxide; *F*, flow-meter; *G*, side-tube for saturating bicarbonate-Ringer with gas mixture; *H*, bottles standing in bath for saturating gases with water vapour, H_L for O_2/CO_2, H_R for N_2/CO_2; *K*, thick-walled rubber tubes connecting with manometers; *MM*, ordinary large Barcroft tank. Stirring, shaking, heating and thermoregulating apparatus not shown. All tubes except *K* are of copper or glass, joined with thick-walled rubber tubing.

Solutions. The medium used throughout was that described by Krebs [1932] containing Na, K, Ca, Mg, Cl, PO_4, SO_4, HCO_3, CO_2, in proportions closely approaching those in serum. Glucose (0·2 %) was added only where mentioned. The medium was saturated with O_2/CO_2 and kept in tonometers. About 260 ml. were prepared at a time and used up within a few weeks.

Lactic acid solution was prepared by dilution of 70 % Na lactate with Krebs medium to give a stock solution of the necessary strength. For pyruvic acid 1 ml. of a 22·5 % solution of redistilled pyruvic acid was brought to p_H 7·6 by the addition of about 2·5 ml. of Krebs medium which had been saturated with $NaHCO_3$. It was then diluted to 10 ml. with Krebs medium. Addition of 0·25 ml. of this to 2·75 ml. of medium in the experiment gave a concentration of roughly 0·02 *N*. All the other substrates used were weighed out and then neutralised and made up to 10 ml. as with the pyruvic acid. With succinate, fumarate, malate and oxaloacetate the normality was calculated on half the molecular weight. In the case of *dl*-malate and *dl*-lactate, experiments were also done with solutions of twice this strength.

Using solutions made up in this way, dilution of the medium was negligible and the only difference from the control experiment consisted in the presence of the substrate and the Na ions necessary to neutralise it.

Oxaloacetic acid was prepared from malic acid by the method of Fenton and Jones [1900], M.P. 144°. Solutions of oxaloacetate had to be made up from the solid immediately before use since in neutral solution this substance appears to decompose to pyruvic acid completely within two days in the ice-box.

General procedure. It was found possible to work simultaneously with three of the Dixon-Keilin apparatus. These were usually prepared the evening before making an experiment, following the instructions of the authors with the added precautions mentioned above.

The animals were stunned by a blow on the back of the neck and bled from the throat. The capsules were at once removed from the kidney, and then slices were cut with a well-sharpened dissecting razor wetted with ordinary Ringer-Locke solution. The surface slice was discarded and it was usually found that

three or four homogeneous slices could be cut from each of several parts of the kidney before the medullary part appeared. Each slice as cut was dropped into a small beaker containing Krebs medium through which O_2/CO_2 was continually bubbled. Sufficient slices for both vessels of one manometer were put into one beaker, the slices necessary for each manometer being kept separate. This precaution was necessary when glycogen estimations were to be made, so that the tissue in two vessels of each manometer was in the medium for about the same length of time. A distributor was used so that gas bubbled simultaneously through each of the beakers containing tissue. All the slices for the three manometers were cut from one kidney.

The well-mixed Krebs medium was then run accurately into both vessels, taking 3, 2·75 or 2·5 ml. according to the addition to be made. The slices, which were handled with flat-bladed coverslip forceps, were rinsed in Ringer-Locke solution, dried gently on smooth filter-paper and weighed. Usually between 110 mg. and 160 mg. wet weight were used, and the amounts placed in the two vessels agreed to 1 or 2 mg. The slices were weighed on a gold wire platform using a specially adapted type of chainomatic balance, which was as rapid as a torsion balance.

In the case of the experiments with minced tissue the whole of both kidneys was minced using a Latapie mincer. It was impossible to weigh the mince into the vessels but it was found very simple to inject a definite volume, 0·3 ml. This was done using a Bashford syringe with the needle attachment removed. This syringe consists of a cylindrical 0·5 ml. barrel calibrated in 0·05 ml. and a glass plunger which goes right to the end. If the plunger is moistened with Ringer solution, the mince can be sucked up out of a crucible, the level accurately adjusted and the desired volume ejected into the medium in the manometer vessel. Two samples are similarly ejected into weighed crucibles and dried for a few hours; their dry weight agrees within 2 mg. in 70 mg.

In the case of lactate experiments the lactate solution was usually run directly into the vessel with an accurate 0·5 ml. pipette. With the other substrates, 0·25 ml. of the solution was measured, using a capillary pipette with its end ground to a point, into danglers, and these were carefully hung in the vessels.

The manometer was joined up and placed in position in the bath and shaking started. When danglers were being used, after passing gas for ten minutes and allowing more than five minutes for equilibration, the manometer stoppers were closed, the apparatus was removed from the bath, acid tipped into the left cup, and then both danglers were shaken down, and the apparatus was replaced in the bath. The remainder of the experiment proceeded as usual. In general we have used an experimental period of 90 minutes before tipping acid into the right cup. Readings were taken after 10, 15 and 30 minutes and every 15 minutes thereafter.

After the final zero reading had been taken, the vessels were removed from the manometers, the side-bulbs and the glass rod, paper and bottom stopper removed and the slices carefully taken out and dropped into about 6 ml. of water in a crystallising dish. The slices were rinsed in two more lots of water and finally placed in a weighed crucible, or, when glycogen estimations were being made, they were put into a weighed centrifuge-tube and covered with about 8 drops of 15 % ammonia. The slices were dried at about 105° for at least 1 hour in crucibles and 3 hours when ammonia was added.

The first wash was added to the vessel contents which were then poured through a funnel into a 25 ml. volumetric flask; the other two washings were

used to rinse out the vessel and the whole made up to 25 ml. This solution was used for the chemical analyses.

Reliability of the manometric measurements. Duplicate experiments given in Table I illustrate the accuracy of the results obtained using this apparatus with the above precautions.

Table I. *Rabbit-kidney cortex in glucose-containing medium.*

	Rabbit no.	Experimental period min.	Wet wt. mg.	Dry wt. mg.	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA} (by estimation)
A	4	60	187	32·3	14·9	0·84	+1·5	+0·86
			101	18·5	14·75	0·85	+1·4	+0·94
B	16	90	85	17·4	13·3	0·83	+1·9	+1·5
			97	19·7	13·6	0·85	+1·5	+1·3

In each case different manometers were in use for the duplicates. In A it should be noted that widely different amounts of tissue were used. In B a considerably smaller amount of tissue was used than was usual, so that small absolute errors give larger proportionate effects.

When working with three manometers it was impossible to start them all simultaneously. About an hour elapsed from the death of the animal until the beginning of the experimental period with the first manometer and a further hour before the start of the last. In order to know what effect this delay might have on the results, the following experiments were done.

Table II. *Rabbit-kidney cortex.*

Experiment	Animal no.	Length of time waiting in bicarbonate-Ringer at room temperature	Dry wt. mg.	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA} (by estimation)
A. Normal, glucose* present	13	½ hr.	25·1	14·4	0·85	+1·6	—
		2 hrs.	28·5	13·0	0·85	+1·7	—
B. Normal, no glucose	12	10 mins.	25·1	12·4	0·70	+0·3	—
		1 hr.	27·3	11·4	0·72	+0·3	—
		2⅓ hrs.	32·5	10·5	0·725	+0·15	—
C. *M*/50 lactate, no glucose present	10	10 mins.	23·2	17·75	0·87	−3·1	—
		2 hrs.	16·0	16·4	0·925	−3·25	—
D. *M*/50 lactate, no glucose	11	10 mins.	22·4	16·5	0·86	−3·8	−3·9
		2⅓ hrs.	31·4	15·45	0·86	−3·4	−3·9

* 0·2 % glucose was present both in the manometer vessel and in the medium in which the tissue waited.

These experiments show that if there is any considerable delay a diminution in respiration of from 3 to 8 % may occur; no conclusions should be drawn when differences as small as this are observed. The R.Q. remains almost unaltered.

In view of these results duplicate experiments were not made, but in each series two experiments with different additions and one without addition were made. Experiments were always repeated completely with tissue from a different animal and often with fresh solutions.

III. Chemical estimations.

Determination of glycogen.

This determination involved two main difficulties. First, there was considerable diffusion of glycogen out of the slices into the medium, so that it was necessary to determine glycogen not only on the tissue itself but also on an aliquot of the manometer fluid; also the slices placed in the two manometer vessels should have been waiting in the bicarbonate medium for about the same length of time. Second, drying the slices in the ordinary way caused a large destruction or hydrolysis of glycogen. Further it was necessary to make sure that no hydrolysis of glycogen occurred during the experiment, since the tissue in the left-hand vessel is shaken in the presence of nearly 0·3 *N* HCl at 38° for up to four hours.

The method of estimation used was in principle that of Pflüger [1909] with suitable modifications. After hydrolysis the Hagedorn-Jensen [1923] method for titration of glucose was used.

Determination in the slices. It was found that by making the slices rather strongly alkaline the loss of glycogen on drying could be avoided. By using 15 % ammonia for this purpose the dry weight was unaffected and could be determined before proceeding with the estimation. After drying and weighing in a centrifuge-tube (18 × 90 mm.), 1 ml. of 60 % (by weight) KOH is added to the tissue, and after fixing on a 12-inch glass tube with a rubber stopper to act as a reflux condenser, the liquid is heated just to boiling on a free flame and the tube placed in a boiling water-bath for 2 hours, shaking occasionally[1]. At the end of this period, 2 ml. of water, 7 ml. of 95 % alcohol and 0·5 ml. of 1 % Na_2SO_4 are added, and the mixture is stirred with a thin glass rod. The latter is finally rinsed with an additional 1 ml. of alcohol. After standing overnight the tube is centrifuged for 10 minutes and the liquid decanted. The precipitate is treated with a further 10 ml. of alcohol (without stirring), and the centrifuging and decanting are repeated. The tube is dried at 110° for 15 minutes to drive off the alcohol which appears to interfere with the Hagedorn-Jensen titration.

The tube is rinsed with 2 ml. of water and 2 ml. of 2 *N* HCl, and heated for 2 hours (with condenser) in a boiling water-bath. After cooling and adding a drop of phenol red[2], the liquid is neutralised with 2 *N* NaOH, the final adjustment to a faint red colour being made with 0·1 *N* NaOH. The solution is poured into a large test-tube and made up with washings from the small tube to a 15 ml. calibration mark. The Hagedorn-Jensen [1923] glucose estimation and blank then follow in the ordinary way, the glucose values being converted into glycogen by multiplication by the factor 0·927.

Determination in the fluid. The vessel contents having been made up to 25 ml., 5 ml. are measured into a centrifuge-tube (18 × 90 mm.), a drop of phenol red is added and *N* NaOH[3] drop by drop until a distinct alkaline reaction is obtained. A cork with glass inlet and exit tubes is inserted and the tube placed in an oven at 110° for evaporation, a gentle current of air being passed through the tube, but not through the liquid, to hasten the evaporation. In practice a distributor and frame were used so that a battery of six tubes could be dried simultaneously. Usually about 3 hours are required.

[1] Since strong alkali causes the tube to lose 4 to 5 mg. in weight, it is necessary to weigh the tube before each determination to obtain the dry weight of tissue.

[2] Alcoholic solutions of phenolphthalein gave high values.

[3] Neutralisation with Na_2CO_3 gave correct results, but 1 drop of 60 % KOH without indicator gave low glycogen values, probably due to destruction by strong alkali present on evaporation.

After the evaporation, 1 ml. of water and 1 ml. of 60 % (by weight) KOH are added, and the mixture is heated for 2 hours under an air-condenser in a water-bath. After cooling 1 ml. of water (not 2 ml. as for the tissue), 7 ml. of 95 % alcohol and 0·5 ml. of 1 % Na_2SO_4 are added and the rest of the procedure is exactly as described for the tissue.

Determination in liver. The above details apply to kidney tissue. Because of the large amount of glycogen in liver it is necessary to change the procedure slightly since the Hagedorn-Jensen micro-method covers only the range 0·002 to 0·385 mg. glucose.

Instead of making up the volume to 15 ml. and oxidising the whole amount, the volume is made up to 25 ml. in a volumetric flask and a suitable aliquot, to be determined by preliminary tests, is oxidised with ferricyanide. Usually 2 to 5 ml. of the solution from the tissue and 5 to 10 ml. of the solution from the vessel fluid are suitable. The proper aliquot is made up to 15 ml. and the usual procedure followed.

Experiments on the glycogen methods.

Estimation of glycogen in solution. Hagedorn-Jensen titrations of solutions containing 0·1 and 0·2 mg. of pure glucose gave 100 % recovery. Similar accuracy was obtained in estimations of pure glycogen solutions. Further, heating 1 ml. of the glycogen solution with 1 ml. of 60 % KOH for 2 hours in a boiling water-bath and carrying the whole procedure through as described above, caused no serious decrease in accuracy. (Required, 0·500 mg. Found: 0·501, 0·501, 0·509 mg.) To test the effect of the long period in the presence of dilute HCl during the manometric experiment, 3 mg. of pure glycogen were dissolved in 6 ml. of bicarbonate Ringer solution and 0·8 ml. of 3·03 *N* HCl were added. (In actual experiments only 0·3 ml. of acid are added to 3 ml. solution.) Samples of 1 ml. were taken at once and after 4 hours' shaking at 38° and the glycogen was estimated, 0·46 and 0·45 mg. being found (taken, 0·45 mg.).

Estimation of glycogen in slices. These experiments were done on slices of rat-liver, usually from unfed animals.

(*a*) The following results show the agreement between estimations of glycogen in slices dropped directly after weighing into 60 % KOH.

	Wet wt. of tissue	mg. glycogen found	mg. glycogen per 100 mg. wet wt.
A	147	1·72	1·17
	172	2·03	1·18
B	173	3·94	2·27
	163	3·66	2·25

(*b*) When tissues are dried after rinsing, but without other treatment, losses of glycogen up to 40 % are observed. These losses are practically eliminated by treatment with 15 % ammonia, a slight loss of 3 % or less would be compensated for since the two sides of the manometer contain equal weights of tissue. Weaker ammonia is not so effective.

Found, mg. glycogen per 100 mg. of wet tissue	
Without drying KOH at once	Drying with 15 % ammonia
7·94	7·76
3·41	3·34

Accuracy of the combined estimations. Rat-liver was sliced in the usual way, and 100 to 200 mg. were placed in 3 ml. of Krebs medium, and 0·4 ml. of 3 *N* HCl was added (*i.e.* slightly more acid than normally). After 4 hours' shaking at 38° the tissue was rinsed and dried with ammonia, and the liquid and rinsing water were made up to 25 ml. A sample of the tissue was dropped immediately into KOH. The glycogen was then determined in the tissue, fluid and the control.

	mg. glycogen found				
	In tissue	In solution	Total	Total per 100 mg. wet wt.	Total per 100 mg. dry wt.
Control* 11 mg. wet	—	—	3·71	3·34	— (15·0)†
Exp. (*a*) 132 mg. wet	2·41	1·60	4·01	3·04	15·2
Exp. (*b*) 164 mg. wet	2·72	2·13	4·85	2·96	14·7

* Fresh tissue dropped directly into KOH.
† Calculated on the wet wt./dry wt. ratio of (*a*) and (*b*).

The estimations after treatment similar to that in an actual experiment agree within 3 % but the results are about 10 % lower than the control and the absolute error is 0·34 mg. In the case of kidney with a lower glycogen content the absolute error is much smaller and the percentage error is also smaller seeing that larger aliquots can be used for titration. Examples of results with kidney appear in the rest of this paper. It is unlikely that the method can be further improved, since determination of the wet weight of the tissue slices cannot be made very accurate without causing damage in the drying on filter-paper, and the dry weights have some uncertainty due to irregular diffusion out of glycogen and other substances and to slight disintegration.

Determination of lactic and pyruvic acids.

For the determination of lactic acid the method of Friedemann *et al.* [1927] as recently modified by Friedemann and Graeser [1933] was followed exactly. Taking a 10 ml. sample (of the 25 ml. of made up manometer fluid) sugars were removed by the copper sulphate-calcium hydroxide method, but as experiments with the Folin-Wu tungstic acid protein precipitant showed that only traces of protein were present, this precipitation was omitted. The sugar precipitation procedure removes all tissue particles and also a part of any proteins in solution.

Since we were making determinations on solutions which in some cases contained succinic, fumaric and malic acids, we tested the extent to which these substances can affect the estimations under the conditions of the method. On titrating a final aliquot of 4/25 of a solution containing 5·3 mg. lactic acid in 25 ml., there were required 9·42 ml. 0·01 *N* iodine. On similar estimation of a solution of the same normality (3·95 mg. in 25 ml.) of malic acid, 2·24 ml. of iodine were required. When the distillation was carried on for 20 mins. instead of 10 mins., 2·44 ml. were needed. With one-fifth of this concentration of malic acid, 0·47 ml. was required. This and other results showed that the titre was roughly proportional to the amount of malic acid and that it gave a titration of about one-fourth of that of an equivalent of lactic acid.

Similar experiments showed no effect of fumarate or succinate on the titration.

Pyruvic acid was estimated on 5 ml. of the made up manometer fluid by the method of Clift and Cook [1932]. Proteins were not removed with trichloroacetic acid, since sufficiently sharp titration end-points were obtained without that

procedure. In the case of anaerobic experiments, where considerable tissue breakdown occurred, and in the experiments with mince, the end-points were less definite.

Results.

In this section results of comparative experiments are presented and interpreted as far as possible. Details of a few typical experiments are shown in the protocols. All the figures were obtained with rabbit-kidney cortex except where otherwise stated. The term "normal" implies that no substrate other than glucose was added. When glucose is stated to have been present, it was present in both the "normal" and in the experiment with substrate addition. The experimental period was in all cases 90 minutes. Except for an unusual experiment which is commented on, all the curves of the movement of the manometer fluid during the experimental period were almost linear, showing that no appreciable changes in the rate of metabolism were occurring. It should be mentioned that complete disappearance of a substrate initially present in 0·02 *N* concentration in 3 ml. of medium would correspond to about 30 M.E. with the weight of tissue slices usually used.

Table III. *Lactate.*

Rabbit-kidney cortex		$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	$Q_{P_{TT}}$	Total glycogen (mg.) in Control cup	Total glycogen (mg.) in Experimental cup	Q_{P_S}
Glucose present	Normal	14·3	0·90	+1·7	+0·8	0·0	0·34	0·63	(+3·0?)
	dl-Lactate (*a*) 0·02 *M* (*b*)	17·7	0·87	−3·1	−3·1	+0·9	0·37	0·34	−0·3
		16·4	0·93	−3·3	−1·3?	+0·5	0·36	0·32	−0·4
No glucose	Normal	12·4	0·76	+0·25	+0·15	0·0	0·33	0·33	0
	dl-Lactate (*a*) 0·02 *M* (*b*)	16·5	0·86	−3·9	−3·9	+0·65	0·37	0·44	+0·5
		15·5	0·86	−3·4	−3·9	+0·45	0·35	0·35	0
No glucose	Normal	11·15	0·76	+0·45	+0·3	0	0·40	0·38	−0·1
	dl-Lactate 0·04 *M*	13·95	0·85	−3·0	−1·65	+0·4	0·38	0·41	+0·2

Lactate (Table III). In the "normal" experiments we see that lactic acid production (Q_{LA}) does not account for the whole acid formation (Q_A). This is more marked in liver, as will be shown in a later paper.

Compared with the other substrates mentioned below, added lactate does not appear to be very rapidly metabolised, even when the amount of lactate is doubled, as was done to give a concentration of the active form comparable with that of substrates mentioned later.

The presence or absence of glucose does not appear to affect the rate of lactic acid breakdown. However in the absence of glucose the R.Q. is, as would be expected, raised by the lactic acid oxidation. The fact that the acid disappearance is as great as or greater than the lactic acid removal would seem to indicate that the lactic acid removed is all oxidised to something other than acid, and the results below make it likely that this means complete oxidation to CO_2 and H_2O.

If this is so we may apply the following consideration. In the first experiment the lactic acid removed in presence of excess is (0·8+3·1=) 3·9 M.E. This amount for complete combustion requires (3·9×3=) 11·7 M.E. of O_2. The oxygen uptake is certainly increased (from 14·3 to 17·7) but not to the extent of 11·7 M.E. Hence it appears that the lactic acid oxidation can replace the oxidation of other substrates, *i.e.* it has a sparing action on their metabolism. The same consideration applies even to those experiments where the lactic acid consumption is smaller.

In these and numerous other experiments the results of the glycogen estimations have convinced us that there is no glycogen synthesis from lactic acid in kidney cortex.

In the above experiments the formation of a small amount of bisulphite-binding substance was observed when lactate was added. This substance seems not to be destroyed by heat in alkaline solution, and we believe it to be pyruvic acid and have entered it as such in the tables. The amount formed is very small, corresponding to a titration of only about 0·12 ml. of 0·002 *N* iodine in a 5 ml. sample of the total 25 ml., after deducting the blank of 0·11 ml. Nevertheless it has been found absolutely invariably when lactic acid was added. Further, practically the whole amount of this small titre is obtained when the experiment is allowed to run for 30 minutes, instead of the usual 90 minutes. We think it probable therefore that the first stage in lactate oxidation in the kidney is to pyruvic acid. This however accumulates only to a slight extent, since, as appears in the next section, pyruvic acid is much more rapidly dealt with than lactic acid.

Table IV. *Pyruvic acid.*

Rabbit-kidney cortex		$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Pyr}	Total glycogen (mg.) in Control cup	Total glycogen (mg.) in Experimental cup	Q_{Ps}
Glucose present	Normal	13·7	0·85	+1·45	+1·3	+0·1	0·33	0·30	−0·2
	Pyruvate 0·016 *M*	14·4	1·17	−3·9	(+2·1)	−7·1	0·28	0·29	+0·1
No glucose	Normal	13·1	0·74	−0·15	+0·2	0	0·26	0·30	+0·2
	Pyruvate 0·017 *M*	15·75	1·07	−5·9	(+1·45)	−7·95	0·30	0·32	+0·2

Pyruvic acid (Table IV). These and other entirely similar experiments show that pyruvic acid is metabolised by kidney cortex about twice as fast as lactic acid (compare *e.g.* $Q_{Pyr} - 7{\cdot}95$ with $Q_{LA} - 3{\cdot}9$), and again the presence or absence of glucose has no important effect. The lactic acid determination shows a certain increase. This may be due to reduction of a portion of the excess pyruvic acid to lactate. It appears probable, however, that it may be partly accounted for by the production of malic acid. It is seen that the oxygen uptake is not increased very much. Considering that only that part of the pyruvic acid disappearance which is accounted for by removal of acid is completely oxidised, we have in the first experiment 3·9 M.E. pyruvic acid oxidised. This requires ($3{\cdot}9 \times 2\frac{1}{2} =$) 9·7 M.E. of O_2. As with lactic acid we see again that pyruvic acid must have a replacing or "sparing" action on other metabolites. Since the R.Q. of the combustion of pyruvic acid is 1·2, the raised R.Q. in its presence is understandable and lends support to the belief that a large amount is completely burnt.

Again there is definitely no indication of the formation of glycogen from pyruvic acid in kidney cortex.

The results of an experiment with rat-kidney cortex are presented in Table V for comparison with the activity of rabbit tissue.

Table V.

Rat-kidney cortex		$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Pyr}
No glucose	Normal	18·6	0·75	+ 0·9	0	0
	With pyruvate 0·012 *M*	27·2	1·24	− 14·2	+5·0	− 18·7

The experimental period in this case was only 60 minutes and small amounts of tissue were used. The same type of result is obtained, except that the tissue from the smaller animal is much more active.

A blank experiment, in which pyruvic acid was present in the medium but no tissue, showed that no spontaneous change in the pyruvic acid occurred, *i.e.* no oxygen uptake, CO_2 output, acid change, change in the titre of pyruvic acid or appearance of "lactic acid" occurred.

The question of acetaldehyde formation. The possibility of decarboxylation of pyrûvic acid in animal tissues has often been discussed. Our experiments convince us that not more than possibly a trace of decarboxylation occurs anaerobically. In three experiments made exactly as normally except that N_2/CO_2 gas mixture was substituted for O_2/CO_2, we obtained respectively $Q_{Pyr} - 1{\cdot}0$, $-0{\cdot}4$, and $0{\cdot}0$, with corresponding amounts of CO_2 evolved. In the aerobic experiments it was possible to show in two ways that no free acetaldehyde was produced. Acetaldehyde boils at 21°, so at 38° its presence should affect the manometric readings. The first method, therefore, consisted in removing the manometer to a bath at 10° to take readings of h_1 and h_2 at both temperatures. Applying the proper manometer constants for these two temperatures it was found that the gas volumes had been unaffected. A separate blank test showed that a small amount of added acetaldehyde affects the manometer strongly above 21° and its effect is completely removed on cooling to 10°. The manometer fluid was washed out and made up with cooled water and the bisulphite-binding substances estimated before and after treatment by alkali and heat. No difference in the small change was found between the solutions from the control and experimental manometer cups so it was again clear that no alkali-labile carbonyl-compound (acetaldehyde) had been formed.

During anaerobic experiments considerable disintegration of the tissue takes place, presumably due to autolysis.

In the anaerobic experiment described in Table VI the calculations were made on the dry weights (29·9 and 25·6 mg.) of the left-hand cups. In the right-hand, experimental, cups the undisintegrated tissues remaining had dry weights of only about half the original.

Table VI. *Anaerobic experiment.*

Rabbit-kidney cortex		Q_{CO_2}	Q_A	Q_{LA}	Q_{Pyr}
No glucose	Normal	0	+0·65	+0·25	0
	Pyruvate 0·015 *N*	0·98	−0·1	+1·2	−1·0

The formation of a little lactic acid from pyruvic acid under anaerobic conditions makes it seem more probable that the "lactic acid" found in the aerobic experiments with pyruvate is at least in part actually lactic acid, produced from pyruvic acid. The reaction lactate $\rightleftharpoons$ pyruvate seems to be to some extent reversible.

Succinate, malate, fumarate and oxaloacetate (Table VII). These are all typical experiments. That these substances are even more rapidly metabolised than is pyruvate can be judged from the high acid disappearance and their effect on the respiratory quotient. The R.Q. of complete combustion for succinate is 1·14, for fumarate and malate it is 1·33 and for oxaloacetate 1·6. The addition of these substrates has raised the total R.Q. considerably, the effects running roughly parallel to the R.Q. of the substrate having regard to the extent of acid disappearance in each case. The results of some lactic acid determinations are shown

Table VII. *Succinate, malate, fumarate and oxaloacetate.*

Rabbit-kidney cortex		$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Pyr}	mg. glycogen in: Control cup	mg. glycogen in: Experimental cup	Q_{Ps}
Glucose present	Normal	11·8	0·88	0	—	0	—	—	—
	Succinate 0·02 *N*	17·2	1·21	−16·9	—	(+1·0)	—	—	—
Glucose absent	Normal	10·4	0·76	− 0·85	+0·2	0	0·39	0·36	−0·2
	Succinate 0·02 *N*	17·0	0·94	−11·6	(+3·4)	(+1·3)	0·40	0·34	−0·4
	l-Malate 0·02 *N*	14·15	1·57	−16·9	(−4·3)	(+1·1)	0·44	0·44	0
Glucose absent	Normal	10·6	0·69	− 0·4	+0·3	0	—	—	—
	dl-Malate 0·04 *N*	14·15	1·44	−13·7	(−5·8)	(+1·25)	—	—	—
Glucose absent	Normal	12·2	0·75	+ 0·6	+0·3	—	—	—	—
	Fumarate 0·02 *N*	15·4	1·42	−15·5	—	(+1·4)	—	—	—
Glucose absent	Normal	11·5	0·76	− 0·65	+0·15	0	—	—	—
	Oxaloacetate 0·02 *N*	14·45	1·54	−11·15	+1·35	−5·6*	—	—	—

* Bisulphite-binding compounds estimated after alkali and heat treatment.

in brackets since it is probable that mainly malic acid was giving the effect in each case and it should be remembered that if it were all malate these figures should be multiplied by about four. With succinate, then, we see a large increase in malic acid which would be due to oxidation of succinate to fumarate, which is then converted to malate. With malate as added substrate, there is a disappearance mainly due to oxidation. With succinate, malate and fumarate definite production of bisulphite-binding substances is found (entered in brackets under Q_{Pyr}). These substances are probably oxaloacetate and pyruvate.

Again we find in the case of all the above substrates the phenomenon of "sparing" the oxidation of other substances. In the case of succinate and malate estimations showed that there was no sign of glycogen synthesis.

As will be seen in the discussion, we consider that the metabolism of pyruvic acid consists in a reaction circuit in which the last step is conversion of oxaloacetic acid back to pyruvic acid.

The results of the experiment with oxaloacetate illustrate this step in the metabolism well. Loss of CO_2 by decarboxylation involves at the same time the disappearance of acid. The acid disappearance measured, $Q_A - 11{\cdot}2$, is then partly made up simply by conversion of oxaloacetic acid into pyruvic acid, and partly by the further combustion of the pyruvic acid. For the estimation of pyruvic acid the solutions were treated with alkali and heated so that all oxaloacetic acid would be converted to pyruvic acid. We see that about 5·6 M.E. of pyruvate have disappeared which would represent mainly that part of the pyruvic acid which is further oxidised. A part, however, is seen to have been reduced to lactate just as pyruvate is reduced when it is added directly as substrate.

Decarboxylation of oxaloacetate does not involve oxidation and, as would be expected, it occurs anaerobically as is shown in the experiment described in Table VIII.

Table VIII. *Anaerobic experiments with 0·02* N *oxaloacetate.*

Rabbit-kidney cortex	Q_{O_2}	Q_{CO_2}	Q_A	Q_{LA}
Glucose absent	0	6·3 to 15·0	− 6·4 to − 15·2	+1·25 to +3·0
Glucose absent	0	15·4 to 20·9	−15·3 to − 20·7	+6·10 to +8·25

In view of considerable anaerobic disintegration of the tissue in the experimental cup the results shown are calculated on the dry weights of the tissue in the left- and right-hand cups respectively. It will be seen later that disintegration of tissue diminishes the activity of the decarboxylating mechanism.

Again we see, as with pyruvic acid added directly, some reduction to lactic acid.

There has been much discussion in the literature concerning the occurrence of carboxylases. These experiments show clearly that there is in intact kidney cortex an active β-decarboxylating mechanism while the α-carboxylase activity is negligible.

Table IX. *Formate.*

	Rabbit-kidney cortex	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Pyr}
No glucose	Normal	12·4	0·76	+1·1	+0·2	0
	Formate 0·02 *N*	12·1	0·76	−0·4	+0·2	0
	Formate 0·02 *N*	11·4	−0·76	+0·1	—	—

Formate (Table IX). Though the R.Q. of formate combustion is 2, its presence is without effect on the R.Q. of the tissue. The slight decrease in acid is nearly within experimental variation. It is clear therefore that formate is not metabolised by kidney cortex to any appreciable extent.

Table X. *Acetate.*

	Rabbit-kidney cortex	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Pyr}
No glucose	Normal	12·2	0·75	+0·65	+0·25	—
	Acetate 0·02 *N*	16·3	0·90	−4·7	+0·25	+0·1

Acetate (Table X). Assuming, as seems reasonable, that the acid disappearance is a rough measure of acetic acid oxidation, it appears that this substance is less actively metabolised than pyruvic, succinic, fumaric, malic and oxaloacetic acids. Its metabolism differs from that of the other substances in that there is no formation of lactate (or malate) or pyruvic acid. Oxidation of 4·7 M.E. of acetic acid involves the uptake of 9·4 M.E. of O_2. Subtracting these amounts from the observed total we get 6·9 M.E. of O_2 taken up by other substrates at R.Q. 0·75. Acetate therefore competes to some extent with other substrates but appears not to alter their type of metabolism since their R.Q. is unchanged.

Table XI. *β-Hydroxybutyrate.*

	Rabbit-kidney cortex	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Pyr}
No glucose	Normal	12·15	0·76	−0·45	+0·2	0
	β-Hydroxybutyrate 0·04 *N*	11·1	0·76	−0·75	+1·4	(+1·7)

β-Hydroxybutyrate (Table XI). This and similar[1] experiments show that the presence of β-hydroxybutyrate has practically no effect on the respiration. There is, however, a definite formation of a bisulphite-binding substance, the amount

[1] One experiment with an old and impure sample of β-hydroxybutyrate showed a rapid formation of acid which gradually came to an end after 60 minutes. It was thought at first to be oxidation to acetic acid, but experiments with pure material did not show this acid formation, so it was probably due to the splitting of polymerisation products.

of which was not altered on treatment with alkali and heat. This substance may be acetoacetic acid, which on the alkali treatment would give acetone. The formation of a substance estimated as lactate is noteworthy. If this actually is lactate it would denote an α-oxidation of the β-hydroxybutyric acid.

In any case this substrate does not behave as a possible intermediary in pyruvic acid breakdown.

Table XII. *Experiments with minced tissue.*

All these experiments were made with minced whole kidneys of rabbits; the dry weight taken was 60–70 mg. No glucose was present in the medium.

Kidney mince	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Pyr}
Normal	1·72	0·62	+0·42	0	0
dl-Lactate 0·03 *N*	1·74	0·62	+0·45	−0·03	+0·02
Pyruvate 0·03 *N*	1·41	0·65	+0·34	(+0·17)	−0·05
Normal	1·84	0·58	+0·38	—	0
dl-Malate 0·04 *N*	2·19	0·64	+0·38	—	(+0·30)
Normal	3·18	0·68	+0·52	+0·97	0·09
l-Malate 0·02 *N*	2·91	0·77	−0·21	(−0·19)	(+0·17)
Fumarate 0·02 *N*	3·62	0·69	+0·25	(+2·23)	(+0·30)
Normal	2·02	0·65	+0·39	+0·13	0
Acetate 0·02 *N*	1·75	0·59	+0·25	+0·06	0
Oxaloacetate 0·02 *N*	1·79	1·64	−1·79	+0·32	−1·0

Experiments with minced tissue (Table XII). These experiments are not exactly comparable with those with slices since whole kidney and not pure cortex was used. Nevertheless cortical matter constitutes a large fraction of the whole organ so that the conclusions drawn seem valid. It is seen that not only is the respiration, as is well known, greatly diminished by mincing but the power to oxidise lactate, pyruvate, malate, fumarate, acetate and oxaloacetate is almost completely abolished. In the case of fumarate we have an increase in the amount estimated by the lactic acid determination and with malate a decrease (which is quite notable compared with the high lactic acid formation in the corresponding normal). These changes, which are fairly large considering that as malate the figures should be multiplied by four, are presumably due to the enzyme fumarase which establishes an equilibrium between fumarate and malate and which seems to retain its activity on mincing. The decarboxylation of oxaloacetic acid is still evident after mincing but occurs at a greatly diminished rate.

The case of succinate is different and interesting. For convenience in discussion the actual volumes, in μl. of gas measured, are included in brackets with the manometric equivalents in Table XIII showing a typical experiment.

Table XIII.

Kidney mince	$-Q_{O_2}$	Q_{CO_2}	R.Q.	Q_A	Q_{LA}	Q_{Pyr}
Normal	2·14 (206)	1·62 (155)	0·75	+0·25	+0·12	0
Succinate 0·02 *N*	5·45 (524)	1·44 (138)	0·26	(< +1·0)	(+1·46) (≡2·26 mg. malate)	+0·23

There is very little change in the CO_2 output and we can apply the following consideration. If, as seems reasonable, the ordinary R.Q. is scarcely changed in

the presence of succinate we would have (138/0·75 =) 184 μl. of O_2 taken up in the ordinary way. This leaves (524 − 184 =) 340 μl. of O_2 extra taken up by the succinate. This corresponds almost exactly with the amount of O_2 (332 μl.) required to oxidise to fumaric acid the whole of the 3·5 mg. of succinate added. The curve of the total gas volume changes during the experimental period (see Fig. 2) indicates a very rapid oxygen uptake which comes to an end after about

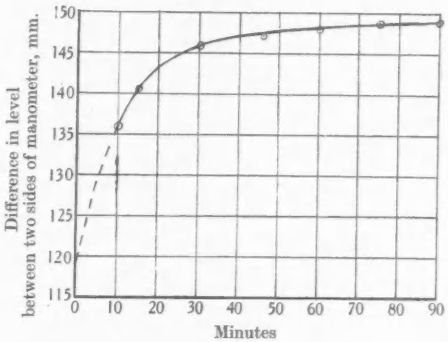


Fig. 2. Minced tissue with succinate. Curve showing change in level of manometer fluid due to resultant of O_2 uptake, respiratory CO_2 evolution and CO_2 displaced or absorbed by acid formation or disappearance.

50 minutes. In view of the rapid change in the curve it is impossible to extrapolate it back to zero time so that the Q_A cannot be estimated properly but it is certainly not negative, *i.e.*, there is no acid disappearance. The lactic acid titration shows an increase of 1·34 M.E.; it is most probable that this increase is all due to malic acid and would then correspond to the formation of about 1·8 mg. malate leaving about 1·7 mg. of fumarate. Slight formation of "pyruvate" is observed due perhaps to a small further metabolism of malate.

It can thus be concluded that in minced tissue the succinic dehydrogenase is still strongly active, as also is fumarase. The further oxidation of malate and fumarate, in confirmation of the previous experiments, is almost completely prevented by mincing.

Protocols.

The following are the details of a typical manometric experiment.

Example 1. *Experiment on kidney cortex slices with pyruvate.*

Medium in Manometer No. 20	3 ml. of glucose-free Krebs bicarbonate medium
Medium in Manometer No. 21	2·75 ml. of glucose-free Krebs bicarbonate medium and in "danglers" 0·25 ml. of the same Ringer containing 4·5 mg. of neutralised pyruvic acid. The danglers were shaken down immediately after turning the acid into the left cup at zero time.
Rabbit killed at 10 a.m	Temperature of bath 38°

Manometer		No. 20	No. 21
Wet weight of tissue in L cup		159 mg.	143 mg.
,, ,, R cup		159 ,,	144 ,,
Zero time		11.15 a.m.	11.50 a.m.
Difference in level of manometer fluid after tipping acid into left cup	10 mins.	−109·0	−119·4
	15 ,,	109·9	120·3
	30 ,,	112·6	123·7
	45 ,,	114·8	126·3
	60 ,,	116·8	129·3
	75 ,,	118·6	131·7
	90 ,,	120·6	133·5
Value of h (obtained by extrapolating curves to zero time)		−13·5	−16·4
Reading h_1. Time after turning acid into R cup (h_1 corrected for final zero)	15 mins.	−13·1	+ 0·1
	25 ,,	−13·1	+ 0·9
		(−13·1)	(+ 0·6)
Reading h_2. Time after turning KOH into both sides (h_2 corrected for final zero)	1½ hrs.	−44·0	−48·7
	2 ,,	43·7	49·0
	2½ ,,	43·8	49·3
		(−43·8)	(−49·6)
Final zero reading		0·0	+ 0·3
Dry weight (w) of tissue in L cup		32·9 mg.	25·4 mg.
,, ,, R cup		29·9 ,,	27·8 ,,
$-Q_{O_2}=\frac{h_2\times k_{O_2}}{w}\times\frac{60}{t}$ mins.		$=\frac{43\cdot8\times13\cdot45}{29\cdot9}\times\frac{60}{90}=13\cdot13$	$\frac{49\cdot6\times13\cdot21}{27\cdot8}\times\frac{60}{90}=15\cdot73$
$Q_{CO_2}=\frac{(h_1-h_2)k_{CO_2}}{w}\times\frac{60}{t}$		$=\frac{30\cdot7\times14\cdot18}{29\cdot9}\times\frac{60}{90}=9\cdot71$	$\frac{50\cdot2\times13\cdot93}{27\cdot8}\times\frac{60}{90}=16\cdot77$
R.Q. $=\frac{Q_{CO_2}}{-Q_{O_2}}$		$=$ 0·74	1·07
$Q_A=\frac{(h-h_1)k_{CO_2}}{w}\times\frac{60}{t}$		$=\frac{-0\cdot4\times14\cdot18}{29\cdot9}\times\frac{60}{90}=-0\cdot13$	$\frac{-17\cdot0\times13\cdot93}{27\cdot8}\times\frac{60}{90}=-5\cdot68$.

Estimations.

The manometer fluids were made up to 25 ml.

Lactic acid. The final aliquot titrated corresponded to 8/25 of the total fluid. A reagent blank of 0·18 ml. has been deducted from all titres.

Manometer cup	Corrected titre ml. of 0·002 N iodine	Lactic acid in total fluid mg.	Difference mg.	CO_2 liberated by lactic acid μl. (1 mg. ≡ 249 μl.)	Q_{LA} $\frac{\mu l.}{w}\times\frac{60}{90}$
20 L	0·22	0·062			
20 R	0·34	0·096	+0·034	+8·5	+0·19
21 L	0·49	0·138			
21 R	1·41	0·396	+0·258	+64·2	+1·43

Pyruvic acid. 5 ml. of the total 25 ml. were titrated. A reagent blank of 0·11 ml. has been deducted from titres.

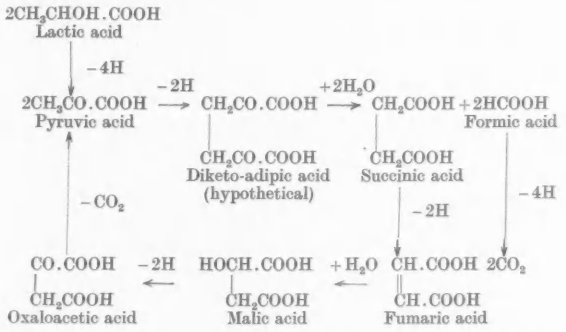
Manometer cup	Corrected titre ml. of 0·002 N iodine	Pyruvic acid in total fluid mg.	Difference mg.	CO_2 liberated by pyruvic acid μl. (1 mg. ≡ 255 μl.)	Q_{Pyr} $\frac{\mu l.}{w}\times\frac{60}{90}$
20 L	0·0	0			
20 R	0·0	0	0	0	0
21 L	10·24	4·51			
21 R	7·49	3·21	−1·30	−332	−7·95

Glycogen. Figures given are in terms of glucose from glycogen.

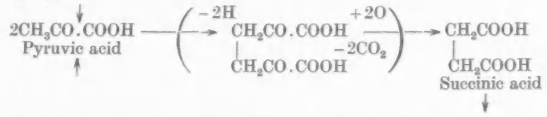
Manometer cup	Glycogen in fluid mg.	Glycogen in tissue mg.	Total glycogen mg.	Difference mg.	Equivalent volume of gas μl. (1 mg. ≡ 249 μl.)	Q_{Ps} $\frac{\mu l.}{w}\times\frac{60}{90}$
20 L	0·19	0·07	0·26			
20 R	0·23	0·07	0·30	+0·04	+10	+0·22
21 L	0·23	0·07	0·30			
21 R	0·25	0·07	0·32	+0·02	+ 5	+0·12

Discussion.

Needham [1932] has summarised the evidence for the following scheme of lactic acid oxidation in muscle.



We feel that a similar scheme explains satisfactorily all the complex series of observations mentioned in this paper. In one particular, however, the scheme does not apply. Since we find no sign of rapid oxidation of formate, the hydrolytic breakdown of the hypothetical $\alpha\alpha'$-diketo-adipic acid apparently does not occur and this section of the scheme must be left more indefinite and should be represented thus:



The fact that lactic acid is first oxidised to pyruvic acid has been indicated frequently in the literature, and our invariable finding of a trace of what is presumably pyruvic acid when lactate is present in excess bears this out. Only a trace is found, however, since, as we find, pyruvate is more rapidly removed than lactate. Succinate, fumarate and malate and oxaloacetate are all even more rapidly removed, and pyruvate, succinate and fumarate all give indications of the formation of malate and traces of keto-compound which may be oxaloacetate or pyruvate which appear later in the cycle.

These substances all appear to "spare" the oxidation of other metabolites, and they appear to be completely oxidised in the end with no sign of glycogen synthesis from them in the kidney.

On destroying the structure of the tissue by fine mincing the mechanism of the cycle appears to be destroyed except for the two stages succinate → fumarate and fumarate → malate. The enzymes mainly responsible for these two steps, succinic dehydrogenase and fumarase remain very active. These facts illustrate the danger of drawing, from experiments on tissues in which the structure is not intact, conclusions such as those of Gözsy and Szent-Györgyi [1934] on the part played by intermediaries. The loss of activity of the enzymes may be due simply to a dilution of the necessary co-enzymes by diffusion from the minced tissue into the medium. Andersson [1934] has shown that while the dehydrogenases of lactate and malate are activated by cozymase or the co-enzyme of Banga *et al.* [1932], succinic dehydrogenase does not seem to require a co-ferment.

Acetic and β-hydroxybutyric acids have also been considered in the literature as possible intermediaries in lactic acid breakdown. Acetate does not appear to be metabolised at quite as high a rate as the above substances, and there seems no place in the scheme where it would occur.

The experiments with β-hydroxybutyrate would indicate that it has no place in the breakdown of lactic and pyruvic acids; rather does it appear to be a possible precursor in lactic acid formation.

Summary.

1. Supplementary and more precise terms for use in describing manometric results are defined.

2. The reliability of the Dixon-Keilin apparatus, when certain precautions are followed, is demonstrated, and a technique is described for using the apparatus to follow the rapid metabolism of various substances by tissue slices and mince.

3. A method for the determination of glycogen changes in tissue slices and medium is described.

4. A study has been made of the metabolism in rabbit-kidney cortex of lactate, pyruvate and various other substances. Measurements have been made of the changes caused by the presence of these substances in determinations of the following: O_2 uptake, R.Q., and total acid, lactic acid, pyruvic acid and glycogen formation or disappearance.

5. Lactate is metabolised slowly by kidney cortex. There is an indication that pyruvic acid is the first stage in its oxidation.

6. Pyruvate is more rapidly oxidised than lactate; and succinate, fumarate, malate, and oxaloacetate are still more rapidly oxidised. In the cases of all these substances there are indications that the one is converted into the other in the foregoing order in the course of its breakdown. Formate is not oxidised.

7. Though the presence of all these substances causes an increase in the rate of respiration, they exert a "sparing" action on the oxidation of other substances present in the tissue. No glycogen is formed from any of them in kidney cortex.

8. Acetate is also fairly rapidly metabolised though there is no suggestion that it is a step in the breakdown of pyruvate. β-Hydroxybutyrate does not affect the respiration appreciably; there are indications that lactate, as well as acetoacetate, is a product of its metabolism.

9. Mincing the tissue almost completely destroys its ability to metabolise the above substances, except that the succinic dehydrogenase and fumarase remain strongly active, and the β-carboxylase acting on oxaloacetic acid remains slightly active.

10. No anaerobic decarboxylation of pyruvic acid occurs in kidney slices, nor can the formation of acetaldehyde be detected in aerobic experiments. Oxaloacetic acid however is decarboxylated to give pyruvic acid.

We are very grateful to Dr Ellice McDonald for his encouragement throughout this work.

[*Note added, October 8th, 1934.*] Krebs has pointed out that in the case of the substances shown in Table VII the total oxygen uptake is not sufficient to account for complete oxidation of the acids disappearing. Also the R.Q. is in some cases higher than could be accounted for by complete oxidation. It would seem that there is another course of metabolism of these substances which also involves disappearance of carboxylic acid. A similar observation was made by Ashford and Holmes [1931] in their study of the oxidation of lactate in brain tissue.

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CCLII. THE ESCAPE OF POTASSIUM FROM RABBIT RED CELLS INTO HYPOTONIC SOLUTIONS.

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Recently it has been shown, by a number of methods, that the volumes attained at equilibrium by mammalian red cells in hypotonic solutions are less than those which would be expected if the cell were a "perfect" osmometer, containing that amount of osmotically active water which it is known to contain and possessing a membrane impermeable to cations [Ponder and Saslow, 1930, 1, 2; 1931; Macleod and Ponder, 1933; Ponder and Robinson, 1934]. This result can be accounted for in several ways, but the best explanation is that a loss of salts occurs from the cell into the hypotonic medium. The extent of this loss, which varies with the way in which the suspension of cells is treated, can be calculated from volume measurements [Ponder and Saslow, 1931], but although both Kerr [1929] and Davson [1934] have shown leakage of Na and K actually to occur, their data are not sufficient to enable us to tell whether the leakage observed is of the right magnitude to account for the volume changes. Further, both Kerr and Davson used ox erythrocytes, whereas the most reliable volume determinations have been made with rabbit cells, and Kerr's determinations of leakage were made after the cells had been standing in hypotonic solutions for several hours. Our idea is, not only that the cells may lose osmotically active substances on standing, but that they do so in the very short space of time during which they are coming to equilibrium with the hypotonic solution. This Jacobs and Parpart [1933] seem to doubt, although they admit the possibility of loss of salts on standing, at least in the case of some kinds of red cell. They believe that they have shown, by an indirect method, that there is very little loss of salts from the ox cell, if, indeed, there is any at all, a point of view which is almost untenable in the face of Kerr's and Davson's analytical data. What is required to make the idea of cation impermeability in hypotonic solutions wholly untenable is a demonstration (*a*) that the quantity of salts lost by the cell, and detected by analysis, is substantially the same as that predicted from the volume measurements, and (*b*) that the volumes from which the calculations are made are attained, not after hours of standing, but within a few seconds. It would then be difficult to escape from the conclusion that the loss of salts occurs during that short time when the cell is swelling to equilibrium. This paper is concerned with such demonstrations.

1. *The loss of potassium.*

In these experiments we have availed ourselves of the fact that the rabbit erythrocyte contains no sodium, and that it can be suspended in NaCl which is K-free. The cations lost from the cell into a hypotonic solution can then be determined as K.

The blood is either oxalated or defibrinated in such a way that no haemolysis occurs (with a glass rod and without touching the sides of the beaker). The

freezing-point of the plasma is obtained by Johlin's [1931] method, and a solution of NaCl of equal freezing-point prepared. With this the cells are washed twice, and a suspension is prepared of about the same volume concentration as that of the original blood. The percentage volume occupied by the cells is found by means of a high speed haematocrit (12,000 r.p.m.). About 10 ml. of this suspension are delivered into a centrifuge-tube and the cells spun down, and the supernatant fluid is drawn up into a small separating funnel, the stem of which has been drawn out into a capillary and turned back upon itself for a short distance at the tip. To this is added enough water to bring the tonicity to about 0·6 % NaCl: the contents of the funnel are then run back into the centrifuge-tube, which is thoroughly shaken. This is the only method which we have found by means of which the tonicity of the suspension medium can be sufficiently reduced without haemolysis occurring. The tonicity of the suspension medium is, of course, a little higher than 0·6 after mixing is complete. The suspension is allowed to stand for 10 minutes.

A second haematocrit determination is now made on this diluted system, and from the two haematocrit determinations the amount of swelling, the exact tonicity, and the apparent quantity of osmotically active water in the cell are calculated [see Ponder and Saslow, 1931; Ponder and Robinson, 1934]. The quantity of water actually present in the cell is determined by weighing packed cells before and after drying, with suitable corrections for the incompleteness of packing [see Macleod and Ponder, 1933]. The cells are thrown down from the rest of the suspension, and the supernatant liquid is drawn off for K determinations. At the same time a quantity of the original suspension (isotonic) is similarly treated: in this way we determine the amount of potassium present in an isotonic NaCl solution surrounding the cells and that present in a hypotonic solution from which the cells have gained water. The method used for determining K was that of Kramer and Tisdall [1921], as described by Peters and Van Slyke [1932].

The results can be expressed in tabular form. The quantities of K lost are expressed as percentages of the total quantity of K present in the cells used in the experiment, assuming each ml. of cells to contain 5·2 mg. of K. In this way the experimental results are most easily compared with the values calculated from Ponder and Saslow's equations. The last column of Table I gives the quantity of K, again expressed as a percentage, lost from the cells into the isotonic NaCl in which the suspension was originally prepared.

Table I.

Tonicity	R	K lost determined	K lost calculated	K lost isotonic NaCl
0·68	0·88	2·1	4·0	0·5
0·68	0·89	4·3	3·5	0·5
0·68	0·77	5·1	7·5	0·3
0·68	0·83	6·8	5·5	—
0·68	0·80	3·0	6·5	—

It is clear that the quantity of K lost by the cells is of the order required by Ponder and Saslow's equations, but there are several points to be specially remarked upon. (1) The R values are rather high, particularly as oxalated blood was used in all the experiments except the first. After a considerable experience with the various kinds of methods used, we think that the R values always tend to be higher when the cell suspension is concentrated than when it is dilute, and that there must be some factor, at present unrecognised, which accounts for this. (2) While the experimental and the calculated values for the K loss are of

the same order, they do not agree very well. The discrepancy is certainly not due to error in the analyses, for all duplicates agreed to within 8 %. There are at least two possible explanations of the discrepancy. The first is that the equations from which the K loss was calculated are based on premises too simple in nature. The second is that the loss of K which occurs at equilibrium may be affected by the leakage of K which occurs even into the isotonic NaCl, this being presumably associated with a Na-K exchange. Davson also has observed that a loss of K occurs (from ox cells) into isotonic NaCl, and even into undiluted serum; if a Na-K exchange actually occurs, the result might well be that the osmotically active substance lost by the cell into a hypotonic solution is not K alone, but also Na, which was not tested for. The initial "isotonic" conditions are, in fact, so much in doubt that we cannot expect a better agreement than has been obtained, particularly as all the methods are being used at the extreme limits of their accuracy.

2. *The rate at which the equilibrium volume is reached.*

In the foregoing experiments, the volumes found were those occupied by the cells after 10 minutes' standing in a hypotonic solution, and it is possible, so far as these experiments alone are concerned, that the salt loss which corresponds to the abnormally small volumes took place gradually over the same length of time. The equations of Ponder and Saslow, however, require that the loss of osmotically active substances shall take place within the time during which the cell is swelling to its equilibrium volume, *i.e.*, that the loss should be very rapid indeed, taking probably only a few seconds. It is obviously impossible to determine K analytically with sufficient rapidity to show that this is so, but we can show that the equilibrium volume from which the extent of the loss of K can be approximately calculated (see above) is attained within 15 seconds, and from this we can infer that the loss of K occurs within this time.

The method is as follows. A series of NaCl solutions is prepared, of such tonicities that tonicities of 0·5, 0·6, 0·7, *etc.*, result when 1 ml. of a suspension of washed cells in isotonic NaCl, of volume concentration about 40 %, is mixed with 6 ml. of the solutions. One such solution is taken and 1 ml. of the suspension added: the mixture is then allowed to stand for 15 minutes. At the end of this time the volume of the cells is measured diffractometrically [Ponder and Saslow, 1931]. After the measurement has been made, the setting of the diffractometer is left unchanged. The rapid measurement requires two persons. One blows from a pipette 1 ml. of the red cell suspension into 6 ml. of the same NaCl solution and then shakes the tube violently. The other takes a small sample from the tube with a capillary pipette and runs a small drop between a slide and a cover-slip, previously placed on the stage of the diffractometer. As quickly as possible, he looks through the telescope at what is at first a field in which no diffraction patterns are visible. As the cells turn into spheres between the slide and cover-glass, the diffraction patterns begin to appear: at first they are indistinct and shifting from smaller angles to larger ones, but they soon assume a brilliant appearance and come to rest. When they do so, they occupy the same position (which the observer can judge by their relation to the cross hair, left in the same position as for the former reading) as those obtained when the cells had stood for 15 minutes in the solution of the same tonicity. As it usually takes about 15 seconds, from the moment of mixing, for sharp patterns to be observed, this means that the volume of the cells is the same after 15 seconds as it is after 15 minutes.

The limiting factors in experiments of this kind are the time which it takes to mix the cells with the hypotonic solution, to transfer the drop to the slide and

cover-glass, and for the cells to assume the spherical form. We have, however, been able to make measurements of the equilibrium volume in as short a time as 13 seconds, and Table II shows the kind of result obtained. The volumes are expressed as percentages of the volume of the cell in isotonic NaCl.

Table II.

Tonicity	Volume 15 mins.	Volume in time t	Time t after mixing, secs.
1·0	100	—	—
0·7	113	113	16
0·6	124	124	13
0·5	137	137	15

The swelling curve obtained by plotting volume against tonicity is one for an "imperfect" osmometer, with an R-value of between 0·5 and 0·6, and the curve is the same whether we measure volume after 15 seconds or after 15 minutes. If, as Jacobs and Parpart are inclined to think, the cells swell as "perfect" osmometers, and the loss of K which can be detected analytically after some 10 minutes occurs gradually as a result of the cells being in a hypotonic solution, we would have the following extraordinary state of affairs. In some time less than 13 seconds, the cells would require to swell to about twice the volumes given in Table II (*e.g.*, in a tonicity of 0·5, to about 165 % of their initial volume), and then they would require to shrink again in order to take up the volumes shown in the table at the end of about 15 seconds. Either during this hypothetical period of shrinking, or thereafter, they would also require to lose the K which is detected analytically in the suspension medium, and yet their volume would have to remain unchanged from 15 seconds after mixing to at least 15 minutes after mixing. It is far more probable that the K is lost in that short time during which the cell is taking in water and assuming its equilibrium volume, and that it is the K lost during this short time which is later detected in the suspension medium.

SUMMARY.

1. When rabbit red cells are suspended in hypotonic NaCl, K is lost from the cells, and the quantity which escapes is of the right magnitude to account for the observed volume changes.

2. The cells reach their equilibrium volume in a hypotonic solution within 15 seconds, and it is presumably within the time during which they are swelling to equilibrium that the loss of K occurs.

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